The PRP2 protein of *Saccharomyces cerevisiae* and its involvement in pre-mRNA splicing

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Declaration

I hereby declare that I alone have composed this thesis and that, except where stated, the work in it is entirely my own.

ABSTRACT

PRP2 protein of *Saccharomyces cerevisiae* is a factor known to be required for pre-mRNA splicing. The aim of this work has been to examine the involvement of PRP2 protein in pre-mRNA splicing, and its interactions with other splicing factors, using a mainly immunological approach.

Antibodies which react with PRP2 protein were raised in rabbits against fusions between different regions of PRP2 protein (which together cover the whole of the protein) and *E. coli* β-galactosidase. These different antisera recognised PRP2 protein with different affinities, under conditions of immunoprecipitation or on western blots. PRP2 protein was over-expressed in yeast using three different expression systems. It was also expressed in *E. coli* and by *in vitro* translation. An assay system for PRP2 protein activity in pre-mRNA splicing was developed (based upon the system described by Lustig *et al*, 1986), and the protein was partially purified by biochemical methods.

Anti-PRP2 antibodies were used to investigate the interactions of PRP2 protein with small nuclear ribonucleoproteins (snRNPs) and with splicing complexes, by studying the RNA species co-precipitated during immunoprecipitations. No association of PRP2 protein with snRNPs was detected. However, it was shown that PRP2 protein is associated with splicing complexes, while temperature sensitive prp2 protein did not associate with such complexes, following heat inactivation. These studies revealed that pre-mRNAs which contain a branchpoint sequence but less than approximately 32 nucleotides downstream of this sequence are preferentially co-precipitated by anti-PRP2 antibodies. Studies of the kinetics of interaction of PRP2 protein with splicing complexes revealed that PRP2 protein associates with complexes at a specific transition between two complexes, and that it remains associated with spliceosomes throughout step 1 of the splicing reaction. PRP2 protein dissociates from the spliceosome prior to step 2 of the splicing reaction, or immediately upon completion of this step. Evidence was obtained that PRP2 protein, in spliceosomes, is associated, either directly or indirectly, with the region of the transcript containing the 5' splice site.

CONTENTS

List of figures

Acknowledgements

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ABBREVIATIONS

A	:	Ampere
ATP	:	adenosine 5'-triphosphate
bp	:	base pair
BSA	:	bovine serum albumin
cDNA	:	complementary DNA
Ci	:	Curie (2.2×10^{12}dpm)
CTP	:	cytosine 5'-triphosphate
D	:	Dalton
dATP	:	2'-deoxyadenosine 5'-triphosphate
dCTP	:	2'-deoxycytosine 5'-triphosphate
dGTP	:	2'-deoxyguanosine 5'-triphosphate
dTTP	:	2'-deoxythymidine 5'-triphosphate
d/ddH_2O	:	distilled/double distilled water
DMSO	:	dimethyl sulphoxide
DNA	:	deoxyribonucleic acid
DNase	:	deoxyribonuclease
dpm	:	distintegrations per minute
DTT	:	dithiothreitol
EDTA	:	ethylene-diamine-tetracetic acid
g	:	accelation due to gravity ($9.81 m.s^{-2}$)
g	:	gram
GTP	:	guanosine 5'-triphosphate
HEPES	:	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
hnRNP	:	heterogeneous nuclear RNP
IAA	:	indole acrylic acid
IgG	:	immunoglobulin G
IPTG	:	isopropyl β-D-thiogalactoside
k	:	kilo-(i.e. $x10^3$)
l	:	litre
m	:	milli-(i.e. $x10^{-3}$)
m	:	metre
M	:	moles/litre

MOPS	:	4-morpholine-propane-sulphonic acid
mRNA	:	messenger RNA
m_3G	:	2,2,7-trimethyl guanosine
M.Wt.	:	molecular weight
n	:	nano-(i.e. $x10^{-9}$)
NP40	:	Nonidet P-40
nt	:	nucleotide
$OD._{600nm}$	:	optical density with respect to light of wavelength 600nm
PAS	:	protein A-sepharose.
PEG	:	polyethylene glycol
PMSF	:	phenyl-methyl-sulphonyl fluoride
Pu	:	purine nucleotide
Py	:	pyrimidine nucleotide
RNA	:	ribonucleic acid
RNase	:	ribonuclease
RNasin	:	ribonuclease inhibitor
RNP	:	ribonucleoprotein
rpm	:	revolutions per minute
rRNA	:	ribosomal RNA
SDS	:	sodium dodecyl sulphate
SDS-PAGE	:	SDS-polyacrylamide gel electrophoresis
snRNA	:	small nuclear RNA
snRNP	:	small nuclear RNP
TCA	:	trichloroacetic acid
TEMED	:	N,N,N',N'-tetramethyl-ethylenediamine
Tris	:	2-amino-2-hydroxymethyl-propane-1,3-diol
tRNA	:	transfer RNA
Tween 20	:	polyoxyethylene(20)-sorbiton-monolaurate
UTP	:	uridine 5'-triphosphate
UV	:	ultra-violet light
V	:	Volt
v/v	:	volume per unit volume
W	:	Watt
w/v	:	weight per unit volume
µ	:	micro- (i.e. $x10^{-6}$)

CHAPTER 1

Introduction

1.1. RNA Splicing

It was discovered in the mid 1970s that the coding regions of many genes are interrupted by stretches of DNA which do not code for RNA sequences found in the mature transcript (Witkowski, 1978). In 1977 it was proposed that these regions (called introns) are transcribed, but are removed from the primary transcript by a process termed RNA splicing. Introns have subsequently been found in eukaryotic genes encoding mRNA, tRNA, rRNA and snRNA, in eubacterial phage genes encoding mRNA and in archaebacterial tRNA genes. Introns are divided into four classes according to the conserved sequences which they contain: for each class of intron a different mechanism of splicing operates.

The different classes of intron are:

(i) introns in nuclear tRNA genes (Abelson, 1979);

(ii) group I introns, in Tetrahymena rRNA, fungal mitochondrial, chloroplast and bacteriophage T4 genes (Cech and Bass, 1986);

(iii) group II introns, in fungal mitochondrial and chloroplast genes (Cech and Bass, (1986);

(iv) pre-mRNA and snRNA introns (Green, 1986; Padgett et al, 1986; Sharp, 1987)

This thesis is concerned with splicing of pre-mRNA introns and the introduction will therefore concentrate on this class of introns. The relationship between different classes of splicing reaction will be discussed in section 1.10.

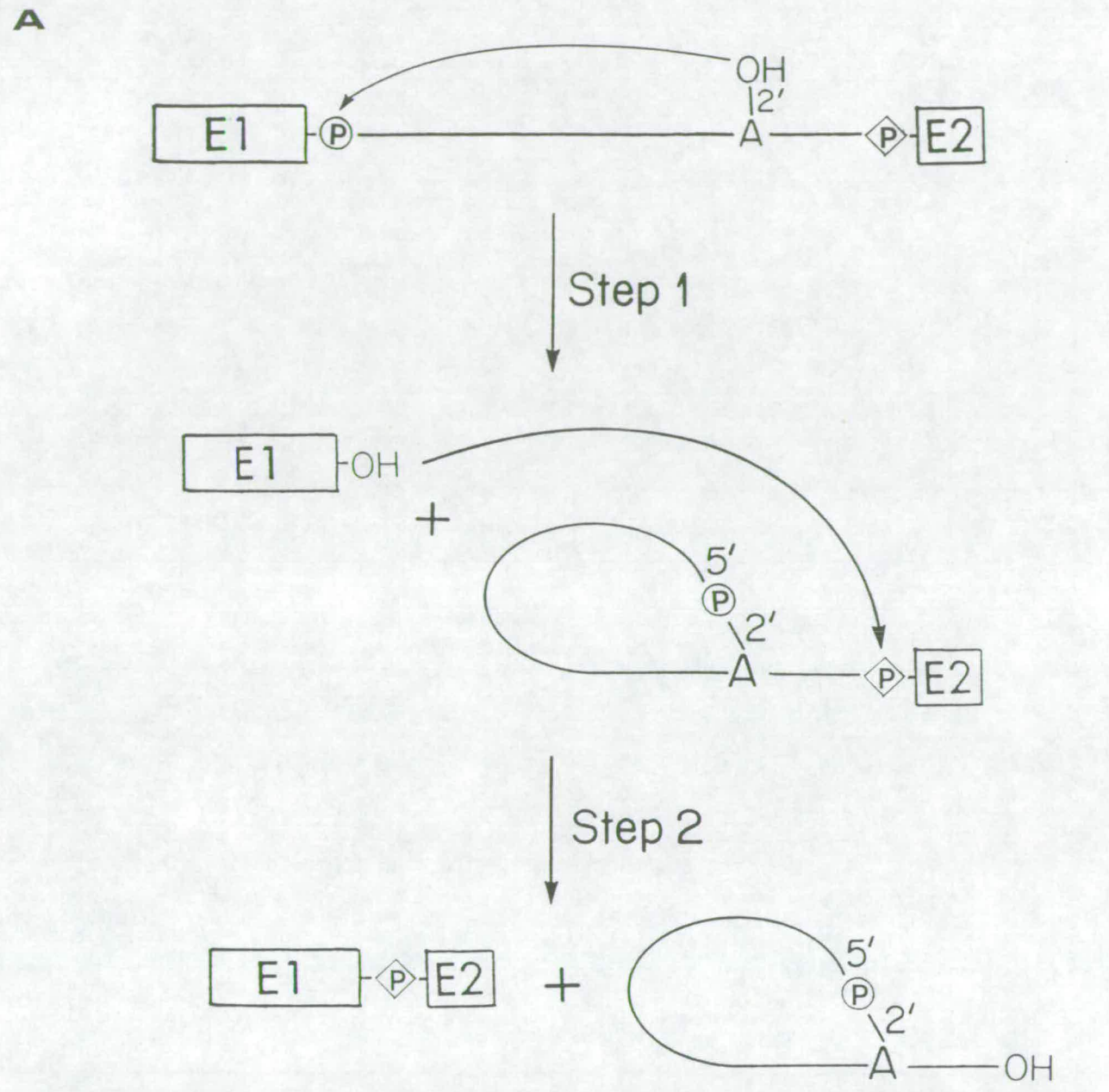
1.2 Structure and Occurrence of Introns

Most higher eukaryotic genes contain introns, and the number of introns in a gene may be as high as 50 (Wozney et al, 1981). These vary in size between 31bp (Ghosh et al, 1978) and approximately 60kbp (Garber et al, 1983; Scott et al 1983), with little conservation of size between analogous introns in related species. By contrast, exon sizes appear to cluster, around means of 52, 140, 223 and 229 base pairs (Naora and Deacon, 1982). In Saccharomyces cerevisiae (henceforth referred to as yeast) few genes have introns, although most ribosomal protein genes do possess one. The largest intron so far discovered in yeast is 513 bp (Leer et al, 1983). Yeast introns are generally found close to the 5' ends of genes.

Comparison of the sequences of yeast and mammalian introns revealed the conserved sequences shown in figure 1. The 5' and 3' splice site consensus sequences are similar in yeast and higher eukaryotes, although yeast introns lack the conserved nucleotides in exon 1 and the polypyrimidine sequence at the 3' splice site. An internal sequence UACUAAC is found in yeast introns at the site of formation of the branch structure in step 1 of the splicing reaction (see section 1.3). This sequence is not found in metazoa, although a less stringently conserved version of the sequence is present in most introns (Keller and Noon, 1984; Keller and Noon, 1985). The sequences at the splice junctions are also more strongly conserved in S. cerevisiae than in metazoa. The introduction of point mutations, and deletion of the conserved sequences have demonstrated that these elements are essential for accurate and efficient splicing (see section 1.4).

1.3 Mechanism of pre-mRNA Splicing

In order to investigate the details of the splicing reaction pathway it was necessary to develop a system in which a single well defined substrate was spliced, and the products and intermediates easily analysed, i.e. an in vitro system. The systems currently used have two main components: a Hela cell nuclear extract, or yeast whole cell extract, and a radioactively-


A
OH
2′
E1
P
A
P
E2
Step 1
E1
OH
+
5′
P
2′
A
P
E2
Step 2
E1
P
E2
+
5′
P
2′
A
OH
B
Metazoa
CAG/GTAAGT
PyTPuAC
Py$_n$NPyAG/G
S. cerevisiae
/GTATGT
TACTAAC
AG/
5′ splice site
branchpoint
3′ splice site

labelled pre-mRNA, produced by transcription *in vitro*, using a bacterial RNA polymerase (Hernandez and Keller, 1983; Hardy *et al*, 1984; Krainer *et al*, 1984; Lin *et al*, 1985). These extracts splice pre-mRNA with an efficiency such that the products and intermediates can be clearly visualised by autoradiography, following polyacrylamide gel electrophoresis.

Development of *in vitro* systems led to the formulation of the two step pathway for pre-mRNA splicing, which is shown in figure 1 (Grabowski *et al*, 1984; Padgett *et al*, 1984; Ruskin *et al*, 1984; Konarska *et al*, 1985a). In the first step of the splicing reaction the pre-mRNA is cleaved at the 5' splice site, leaving a 3' hydroxyl group on exon 1. The first residue of the intron forms an unusual 2'-5' phosphodiester bond with an adenosine residue near the 3' end of the intron, creating a branched or 'lariat' structure. In yeast this adenosine is the most 3' adenosine of the UACUAAC sequence (Domdey *et al*, 1984; Rodriguez *et al*, 1984).

These intermediates are converted into products in the second step of the reaction. In this step the lariat intermediate is cleaved at the 3' splice site and the exons ligated, resulting in two products: the spliced mRNA and the excised intron, (which remains in the form of a lariat), with a 3' hydroxyl group.

Although ATP is necessary for *in vitro* splicing, the number of phosphodiester bonds is conserved in the products and therefore the reaction could theoretically proceed without any energy imput. It is believed that both steps of the reaction proceed by trans-esterification reactions (e.g. in step 1 by direct nucleophilic attack by the 2' hydroxyl of the branchpoint adenosine upon the phosphate at the 5' splice site), since it has been impossible to detect cleavage of the RNA in either step of the reaction in the absence of the concomitant ligation (2'-5' or exon 1-exon 2). One of the requirements for ATP in *in vitro* splicing is in the assembly of a large complex in which the reaction takes place (see section 1.7).

The requirements of the *in vitro* splicing reaction for ions and cofactors appear to be similar in yeast and mammalian systems. In both,

monovalent cation (Na^+, K^+) concentrations of 50-100 mM are optimal (Lin *et al*, 1985; Hernandez and Keller, 1983; Krainer *et al*, 1984). There is a requirement for low levels of Mg^{2+}, while high levels inhibit splicing. ATP is also necessary, and analogues in which either the α-β or β-γ bond is non-hydrolysable do not support splicing (Krainer *et al*, 1984; Lin *et al*, 1985). In neither the mammalian nor yeast system is it necessary for the RNA to be 5'-capped, although this does improve splicing efficiency in the former (Krainer *et al*, 1984; Inoue *et al*, 1989). The temperature optimum, *in vitro*, is 30°C for the mammalian system and 25°C for yeast.

That the same reaction pathway occurs *in vivo* as *in vitro* has been confirmed by detection of branched molecules in HeLa cell heterogeneous nuclear RNA (hnRNA) (Wallace and Edmonds, 1983) and by the identification of lariats in *in vivo* β-globin transcripts, some of which were identical to those produced during *in vitro* splicing of the β-globin transcript (Zeitlin and Efstratiadis, 1984; Ruskin *et al*, 1984). Similar experiments with *S. cerevisiae* have confirmed that the pathway is the same *in vivo* and *in vitro* (Domdey *et al*, 1984; Rodriguez *et al*, 1984; Lin *et al*, 1985).

1.4 Pre-mRNA Sequences Required for Splicing

1.4.1 Mammalian pre-mRNA

As indicated in section 1.2, the only conserved sequences in mammalian introns are at the 5' and 3' splice sites and at the weakly conserved branch point sequence, suggesting that it is only these sequences which are required for splicing. Deletion studies have shown that within the intron, only 6 nucleotides at the 5' splice site and 24 nucleotides at the 3' splice site are essential for splicing, provided a minimum intron size of 80 nucleotides is maintained (Wieringa *et al*, 1984; Van Santen and Spritz, 1985). There is no apparent upper limit on intron size. Removal of all but 20 nucleotides of exon 1 and 4 nucleotides of exon 2 permits splicing (Parent *et al*, 1987; Turnbull-Ross *et al*, 1988); exon 2 is not absolutely required for step 1 of the reaction (Frendewey and Keller, 1985). However,

the severity of the effect of truncating exon sequences may vary in different genes.

At the 5' splice site in β-globin genes, naturally occurring mutations inactivate the splice site, causing activation of cryptic 5' splice sites. This produces abberrant, or truncated, β-globins and therefore results in thalassemias (Treisman et al, 1983). The cryptic 5' splice sites which are used generally have strong homology to the 5' splice site consensus sequence (Krainer et al, 1984). Mutation of G1 or U2 inhibits cleavage to varying degrees, and may permit step 1 of the reaction, but not step 2 (Aebi et al, 1987). Purine transitions at intron positions 3 and 4 have little effect on splicing (Treisman et al, 1983), while changes at position 5 often lead to the utilization of cryptic 5' splice sites (Wieringa et al, 1983).

Due to the lack of strong conservation of mammalian branchpoint sequences, and the finding that deletion of authentic branchpoints led to activation of cryptic branchpoints, with little effect upon the efficiency of in vitro splicing (Ruskin et al, 1985), it was believed that the sequence surrounding the branchpoint nucleotide was relatively unimportant. Branches can form at G and U nucleotides, but these are blocked for step 2 of the reaction (Aebi et al, 1987). However, two recent studies, using similar cis-competition assays have revealed that branchpoint sequence mutants can strongly affect splicing efficiency, both in vitro and in vivo (Zhuang and Weiner, 1989a; Reed and Maniatis, 1988). It was also shown that when two branchpoint sites within the same intron are in competition, the match of the branchpoint sequences to the mammalian consensus sequence YURAC is an important determinant of the efficiency of utilization of a particular branchpoint. These competition assays demonstrated that the yeast consensus sequence UACUAAC is the most efficiently utilized mammalian branchpoint sequence (Zhuang et al, 1989a). In general, the severity of effect of mutations in the mammalian branchpoint sequence parallels the effect of analogous mutations in the yeast branchpoint sequences (Reed and Maniatis, 1988).

The branchpoint nucleotide is usually 18-40 nucleotides upstream of the 3' splice site (Ruskin et al, 1984; Keller and Noon, 1984; Reed and Maniatis, 1985), and it was assumed until recently that there is a strong distance constraint upon the position of the branchpoint. While the polypyrimidine tract has been shown to be essential for step 1 of the reaction (Frendewey and Keller, 1985; Reed and Maniatis, 1985; Ruskin and Green, 1985; Reed, 1989), mutations in the AG dinucleotide have effects varying from nil to almost complete abolition of step 1, in different genes (Reed and Maniatis, 1985; Ruskin and Green, 1985; Aebi et al, 1986; Smith et al 1985). Recent studies (Reed, 1989; Smith et al, 1989) have shown that branchpoints up to 177 nucleotides upstream of the 3' splice site can be active in splicing, provided that they are adjacent to a long polypyrimidine tract. However, in most genes the short polypyrimidine tract results in a requirement for the AG dinucleotide for efficient lariat formation (Reed, 1989). The AG dinucleotide is essential for the second step of splicing (Reed and Maniatis, 1985; Ruskin and Green, 1985; Aebi et al, 1986). It appears that the 3' splice site is the first AG downstream of the branchpoint (Reed and Maniatis, 1985; Smith et al, 1989).

1.4.2 Yeast pre-mRNA

The yeast splice site and branchpoint consensus sequences are more strongly conserved than those in metazoa, and deletion of 5' splice site and branchpoint sequences does not, in general, lead to utilization of cryptic sites in yeast. This may reflect a greater degree of sequence specificity of the yeast splicing machinery. Since metazoan introns do not contain the UACUAAC consensus sequence, they are not spliced, or abberrantly spliced in yeast (Watts et al, 1984). The yeast RP51A pre-mRNA can be spliced in a HeLa extract, although a different branchpoint is used (Ruskin et al, 1986).

As with mammalian introns, the bulk of yeast intron sequences, with the exception of the consensus sequences is not required for splicing. The two smallest naturally occurring introns, in the MATa1 gene have 42 and 43 nucleotides separating the 5' splice site and branch point (Miller, 1984), which is close to the minimum, (44 nucleotides) determined by deletion

studies (Thomson-Jager and Domdey, 1987). The average size of yeast introns is 300-500 nucleotides and large insertions decrease the efficiency of splicing (Klinz and Gallwitz, 1985). Most introns are positioned near the 5' end of transcripts and moving an intron downstream resulted in a decrease in splicing efficiency (Klinz and Gallwitz, 1985). A first exon as small as 12 nucleotides permits splicing, while one consisting of 1 nucleotide permits step 1 of the reaction (Duchene et al, 1988). Exon 2 is dispensible for step 1 of the reaction, and a second exon of 10 nucleotides is sufficient for splicing (Rymond and Rosbash, 1985).

In contrast to the situation in metazoa, deletion of the 5' splice site blocks splicing (Gallwitz, 1982). Point mutations at position 1 and 2 of the intron completely inhibit step 2 of the splicing reaction and with the exception of G1→A1 also inhibit step 1 (Newman et al, 1985; Fouser and Friesen, 1986; Vijayraghavan et al, 1986). The G residue at position 5 appears to be important for selection of the cleavage site in step 1, since mutation to A or C permits a low level of cleavage at cryptic sites, which, interestingly, bear no sequence resemblance to the 5' splice site consensus (Fouser and Friesen, 1986; Vijayraghavan et al, 1986; Parker and Guthrie, 1985). This suggests that the 5' splice site cleavage activity in step 1 of splicing may not be strictly dependent on RNA sequence.

Deletion of the branch point sequence abolishes splicing in all cases tested, except that of actin, where a cryptic branch site, close to the normal branchpoint is used (Langford and Gallwitz, 1983; Langford et al, 1984; Pikielny et al, 1983; Cellini et al, 1986). Point mutations in the branchpoint sequence have variable effects upon the two steps of splicing, sometimes depending on the nucleotide introduced, and possibly also on different genes and detection methods employed (Newman et al, 1985; Fouser and Friesen, 1986; Vijayraghavan et al, 1986; Langford et al, 1984; Jacquier and Rosbash, 1986; Jacquier et al, 1985). The branchpoint nucleotide is always at position 6 in the sequence, but the presence of an adenosine at this position is more important for step 2 of the reaction than step 1. Branches can form when other nucleotides are present at this position, but these strongly inhibit step 2 of the reaction. It has been suggested that the stricter dependence of step 2 than step 1 on the 5' splice site G1 and

branchpoint A6, (the two nucleotides joined at the branch), may constitute a proof-reading step in splicing. While mutations at some branchpoint sequence nucleotides can have drastic effects on splicing, mutants at other positions e.g. U1 and A2 seem to have little effect (Fouser and Friesen, 1986; Langford et al, 1984; Jacquier et al, 1985). This seems paradoxical given the absolute conservation of these nucleotides in yeast introns, but may reflect laboratory growth conditions which may not be suitable for testing the phenotypes of these mutations.

If the 3' splice site AG is deleted in yeast, the first AG downstream of the branchpoint is utilized (Langford and Gallwitz, 1983). Transcripts which lack any AG undergo step 1 of the splicing reaction efficiently, provided that they contain more than 29 nucleotides 3' to the branchpoint sequence (Rymond et al, 1987). Mutation of AG to AC strongly inhibits step 2 of the reaction (Vijayraghavan et al, 1986).

In addition to conserved sequences, studies of individual genes have revealed non-conserved sequences which affect the efficiency of splicing of particular pre-mRNAs. Newman (1987) identified a region upstream of the TACTAAC sequence in the CYH2 gene which was required for efficient splicing. In genes in which this sequence was deleted, efficient splicing could be restored by deletion of another element, close to the 5' splice site, suggesting the presence of an inhibitory sequence in this region. Another study identified a region near the 5' splice site in the RP51A intron, which was responsible for increasing the efficiency of splicing of this pre-mRNA (Pikielny and Rosbash, 1985).

1.5 Trans-acting Factors Involved in pre-mRNA Splicing

The second main focus of research on pre-mRNA splicing has been the identification of the trans-acting factors which comprise the splicing machinery and investigation of the role of these factors in splicing. These factors may be divided into two main categories: (a) small nuclear ribonucleoprotein particles (snRNPs) and (b) other protein factors.

1.5 Mammalian snRNPs

1.5.1 Mammalian snRNAs

Small nuclear ribonucleoprotein particles are found in the nuclei of eukaryotic cells and consist of small RNA species, stably associated with proteins (Steitz, 1988). The snRNAs which are involved in RNA splicing are U1, U2, U4, U5 and U6 RNAs. These are uridine rich, and are the most highly abundant small nuclear RNAs ($2x10^5$-$1x10^6$ copies/cell). They range in size from 106 to 189 nucleotides and with the exception of U6, the cap structure of which is unknown, possess a trimethyl guanosine (m_3G) cap at their 5' end. Certain nucleotides are modified in U snRNAs but the functional role of these modificiations is uncertain. The U1, U2 and U5 RNAs are each contained within a separate paricle, while U4 and U6 RNAs are base paired to each other in a single snRNP (Hashimoto and Steitz, 1984; Bringmann et al, 1984; Rinke et al, 1985).

The U1-6 snRNAs all contain extensive secondary structure, although in different species some of these features may be deleted or severely reduced. U6 RNA is the most strongly conserved RNA, although different regions of the RNAs are conserved to different degrees, and this has provided valuable evidence on the importance of these regions in snRNA function (see Guthrie and Patterson, 1988 for a review). Extensive intermolecular base pairing between U4 and U6 has been confirmed by the consistent finding of complementary base substitutions in different species. U1, U2, U4 and U5 all contain a motif which contains the sequence $PuAU_nGPu$ ($n>3$), flanked by double stranded stems (Branlant et al, 1982). This conserved sequence is a binding site for Sm antigens (see section 1.5.1.2).

1.5.1.2 Mammalian snRNP proteins

There are several autoimmune disorders in humans, in which snRNPs act as autoantigens, and sera from patients with these disorders react with all the snRNPs (anti-Sm), with a subset (anti-(U1,U2)-RNP) or with individual snRNPs (anti-U1-RNP, anti-U2-RNP) (Van Venrooij, 1987). SnRNPs have been immunopurified using these sera and anti-m_3G antibodies (Hinterberger et

al, 1983; Kinlaw et al, 1983; Bringmann and Luhrmann, 1986). The U snRNPs all contain a common set of 7 proteins (B, B', D, D', E, F and G). cDNAs encoding D and E proteins have been cloned (Rokeach et al, 1988; Wieben et al, 1985). The B and B' proteins show immunological cross-reactivity and similarity in their peptide maps (Reuter et al, 1987).

Evidence from in vivo pulse labelling experiments suggests that the D, E, F and G 'core' proteins form a 6S cytoplasmic complex prior to binding to snRNAs (Fisher et al, 1985). These proteins are tightly bound to the Sm binding site after prolonged micrococcal nuclease digestion of snRNPs (Liautard et al, 1982), although only F can be UV cross-linked to the snRNA (Woppmann et al, 1988). The B and B' proteins have recently been shown to have a nuclease activity which is not sequence specific, and is inactive in native snRNPs (Temsamani et al, 1989). The function of this activity and its role in RNA splicing is uncertain.

The U1 snRNP contains 3 specific proteins, A, 70K and C. cDNAs encoding these proteins have been cloned (Thiessen et al, 1986; Sillekens et al, 1987, 1988; Spritz et al, 1988) . The sequence of the cDNAs encoding A and 70K reveals that they, like the U2 specific protein B" (Habets et al, 1987) both contain an 80 amino acid domain which is common to a number of RNA binding proteins (Dreyfuss et al, 1988). In the case of the 70K protein, this has been shown to be part of a 110 amino acid RNA-binding domain (Query et al, 1989). In vitro reconstitution studies of U1 snRNP have shown that after the DEF and G proteins have bound to the Sm site, 70K and A both bind to stem-loop 1 of U1 RNA, and this binding is stabilized by the binding of the DEF and G 'core' proteins (Patton et al, 1987; Patton and Pederson, 1988; Hamm et al, 1988). C protein appears to be required for stabilization of the base pairing interaction between the 5' splice site and U1 RNA (Heinrichs et al, 1990) (see section 1.5.3.1).

The U2 snRNP contains 2 specific proteins, A' and B", and cDNAs encoding these proteins have been cloned (Sillekens et al, 1989; Habets et al, 1987). These proteins require the 3' terminal stem loop of U2 RNA in order to bind (Mattaj and De Robertis, 1985). However it appears that they are not

required for the activity of U2 snRNP in splicing (Pan and Prives, 1989; Hamm et al, 1989).

Recently, it has been shown that a fraction of U5 snRNPs contain, in addition to the 7 common proteins, up to 7 unique proteins (Bach et al, 1989). Two independent studies have identified a protein of 70K or 100K (Tazi et al, 1986; Gerke and Steitz, 1986) which is an Sm antigen, associated with U5 snRNP. This protein is known as intron binding protein (IBP), since it binds to the polypyrimidine tract at the 3' end of introns, independently of U5 snRNP, at 0°C and in the absence of ATP. U5 snRNP also contains a >200kD protein which appears to be the mammalian homologue of the yeast PRP8 protein (Anderson et al; 1989 Pinto and Steitz, 1989)

1.5.2 Yeast snRNPs

1.5.2.1 Yeast snRNAs

In yeast, snRNAs are much less abundant than the U1-6 RNAs of mammals, and are present at less than 200 copies per cell (Wise et al, 1983; Riedel et al, 1986). This may reflect the infrequency of introns in yeast genes. They have the same biochemical properties, of stability, possession of a 5' m_3G cap and modified nucleotides. Initial studies identified 24 such snRNAs, of which 5 of the first 6 cloned were dispensible for cell growth (Parker et al, 1988; Tollervey et al, 1983; Tollervey and Guthrie, 1985). The homologues of the mammalian U1, 2, 4, 5 and 6 RNAs (previously SNR 19, 20, 14, 7 and 6, respectively) have now been identified (Ares, 1986; Kretzner et al, 1987; Siliciano et al, 1987a, b; Patterson and Guthrie, 1987). They all contain the Sm binding consensus sequence, with the exception of U6.

Yeast U1 snRNA is 568 nucleotides in length and contains several large insertions, relative to the mammalian U1 RNA (Kretzner et al, 1987; Siliciano et al, 1987b). It lacks stem-loops 2 and 3 in the consensus U1-RNA structure, but is identical to mammalian U1-RNA over its first ten nucleotides. Yeast U2 RNA is even larger at 1175 nucleotides, but contains

a 950 nucleotide region which may be deleted without apparent effect on cell viability (Igel and Ares, 1988; Shuster and Guthrie, 1988). It also contains a sequence GUAGUA which is complementary to the yeast intron branchpoint sequence. U4 and U6 RNAs are more similar in size to their mammalian counterparts, but U6 is far more strongly conserved (Brow and Guthrie, 1988). They have been shown to be base paired to each other in the same snRNP (Brow and Guthrie, 1988), although the melting temperature of the U4/U6 hybrid is anomalously high.

Yeast U5 RNA exists in two forms which are present in equal amounts. These differ by the presence of an extra 35 nucleotides at the 3' end of the larger form. The predicted secondary structure of this RNA is similar to the mammalian species, and a 9 nucleotide sequence, which is predicted to be in a single stranded loop is absolutely conserved.

1.5.2.2 Yeast snRNP proteins

Using a genetic approach to the identification of splicing factors (section 1.6.2), it has been possible to identify yeast snRNP proteins. Thus, the PRP8 protein is associated with U5 snRNP (Lossky et al, 1987) while PRP4 protein is associated with U4/6 snRNP (Banroques and Abelson, 1989; Peterson-Bjorn et al, 1989). In addition to these, yeast U1, 2, 4, 5 and 6 RNAs are associated with homologues of the mammalian Sm antigens, since they can be precipitated from in vitro splicing extracts using these sera (Tollervey and Mattaj, 1987), or following injection of the RNAs into Xenopus oocytes (Tollervey and Mattaj, 1987; Riedel et al, 1987).

1.5.3 Role of snRNPs in splicing

1.5.3.1 Mammalian systems

The initial suggestion of a role for snRNPs in RNA splicing was based on the complementarity of the first 10 nucleotides at the 5' end of U1 RNA to the 5' splice site consensus sequence (Lerner et al, 1980; Rogers and

Wall, 1980). Subsequent studies showing that anti-Sm and anti-U1-RNP antibodies (Yang et al, 1981; Padgett et al, 1983) and oligonucleotide-directed cleavage of the 5' end of U1 RNA (Krainer and Maniatis, 1985) both inhibited splicing, confirmed this hypothesis with respect to U1 RNA. It was also shown that U1 snRNP, but not the RNA alone, protects the 5' splice site region of pre-mRNA from digestion with T1 RNase (Mount et al, 1983). U1 snRNP also binds to cryptic 5' splice sites (Chabot and Steitz, 1987a) and to synthetic 5' splice sites, which have been inserted into a pre-mRNA (Nelson and Green, 1988).

It has been directly demonstrated, by suppression of 5' splice mutations by the complementary mutation in U1 RNA, that U1 RNA does interact with the 5' splice site through Watson-Crick base pairing (Zhuang and Weiner, 1986). This base pairing interaction seems to determine the exact site of 5' cleavages, since it was possible to predict the site of cleavage in different mutants on the basis of the strength of base pairing interactions with U1 RNA (Aebi et al, 1987; Weber and Aebi, 1988). Comparison of several thousand splice site sequences suggested that although the hybridizing region of the pre-mRNA is composed of two domains, (one of which is dispensible provided the overall stability of the hybrid is maintained), cleavage always occurs opposite the bond between residues 9 and 10 of U1 RNA (Jacob and Gallinaro, 1989) .

Oligonucleotide directed cleavage of U2 RNA demonstrated the involvement of U2 snRNP in splicing (Black et al, 1985; Krainer and Maniatis, 1985). Precipitation of protected T1 RNase fragments from the branchpoint region with anti-U2-RNP indicated that U2 snRNP was bound to this region during splicing (Black et al, 1985). It has recently been shown that the effects of branchpoint sequence mutations can be suppressed by the complementary mutations in the GUAGUA sequence in U2 RNA (Wu and Manley, 1989; Zhuang and Weiner, 1989b). Thus, U2 snRNP interacts with the branchpoint sequence through Watson-Crick base pairing at some stage of the reaction. In order to accomodate the observed variability of branchpoint sequences, it has been proposed that overlapping domains of U2 RNA base pair with the branchpoint sequence (Hartmuth and Barta, 1988). It has been suggested that in this base pairing scheme, the branchpoint adenosine may be

unpaired, or 'bulged', in a comparable way to the proposed mechanism for splicing of group II introns (Cech and Bass, 1986). This arrangement may facilitate nucleophilic attack by the 2' hydroxyl group of the branchpoint adenosine upon the phosphate group at the 5' splice site.

The involvement of U5 RNA in splicing has not been demonstrated by oligonucleotide directed cleavage, due to the resistance of U5 snRNP to cleavage using this approach. However, as noted above (section 1.5.1.2), U5 is associated with a protein which binds to the 3' splice site region. The 3' splice site region was shown, in a separate study (Chabot et al, 1985), to be protected by an m_3G precipitable, but micrococcal nuclease resistant factor. Of the major snRNPs only U5 shows this degree of resistance to micrococcal nuclease. Recently, it has been shown that extracts depleted of U5 snRNP are able to undergo step 1 of the splicing reaction but not step 2, although the efficiency of step 1 was also inhibited (Winkelmann et al, 1989). Thus U5 may only be absolutely required for the second step of pre-mRNA splicing.

Although U4/6 is required for splicing, as shown by oligonucleotide-directed cleavage experiments (Berget and Robberson, 1986; Black and Steitz, 1986), no direct binding of the U4/6 snRNP to the RNA has been demonstrated.

1.5.3.2 Yeast systems

Disruption of the genes encoding the yeast U snRNA homologues has shown that they are all required for cell viability (Ares, 1986; Kretzner et al, 1987; Siliciano et al, 1987a, b; Brow and Guthrie, 1988; Patterson and Guthrie, 1987). A yeast strain containing a conditional null allele of SNR7 was constructed by placing this gene under the control of the glucose-repressible GAL1 promoter (Patterson and Guthrie, 1987). After growth on glucose for a number of generations, the level of U5 snRNA was reduced to less than 0.5% of the normal level, and this resulted in inhibition of pre-mRNA splicing. Interestingly, and in parallel with the finding in a mammalian system (Winkelmann et al, 1989), for some transcripts tested, U5 depletion resulted in the accumulation of lariat intermediates. This

result suggests that normal levels of U5 snRNP are only absolutely required for the second step of splicing.

Similar genetic experiments to those performed on mammalian U1 RNA showed that yeast U1 RNA interacts with the 5' splice site through Watson-Crick base pairing (Seraphin et al, 1988; Siliciano and Guthrie, 1988). By the same technique (Parker et al, 1987) it was demonstrated that base pairing occurs between a GUAGUA sequence in U2 RNA and the UACUAAC sequence in yeast introns.

1.6 Other Trans-acting Factors

1.6.1 Mammalian splicing factors

Both in vitro and in vivo, pre-mRNA is complexed with heterogeneous nuclear ribonucleoprotein (hnRNP) proteins (Dreyfuss et al, 1988). It was initially thought that these proteins had no sequence specificity of RNA binding, but recent work has shown that the hnRNP proteins C, A1 and D bind specifically to sequences near the 3' end of introns, including the polypyrimidine tract and branch point sequences (Swanson and Dreyfuss, 1988a,b). Depletion of the hnRNP C protein from extracts showed that it is required for splicing (Choi et al, 1986). It has been suggested that a relatively high non-specific RNA binding affinity in these proteins allows them to find their target sequences rapidly, presumably while the RNA is being transcribed, by binding to the RNA and diffusing in one dimension only.

A variety of soluble protein factors have been identified by fractionation of HeLa cell extracts (Kramer and Keller, 1985; Kramer et al, 1987). Some of these factors are required only for the second step of splicing.

A factor that is necessary for U2 binding to pre-mRNA, but which binds independently from it, was identified (Ruskin et al, 1988). This protein, U2AF, requires both the 3' splice site dinucleotide and the polypyrmidine

tract in order to bind efficiently, and was shown to be distinct from hnRNP C protein and the U5-associated intron binding protein.

Another protein, of molecular weight 62kD, which also binds to the polypyrimidine tract, has recently been purified (Garcia-Blanco et al, 1989).

There is evidence for the existence of a factor which collaborates with U1 snRNP binding to 5' splice site sequences. Using a filter binding assay Mayeda et al (1986) detected an activity in the flowthrough from a U1 snRNP purification column which stimulated U1 binding to an RNA substrate containing only a 5' splice site. Recently, by simply pelleting snRNPs by ultracentrifugation a factor was detected which allowed U1 to protect the 5' splice site (Zapp and Berget, 1989). Complexes containing U1 and U2 snRNP and pre-mRNA formed in the absence of this factor, but the 5' splice site was not protected in such complexes.

1.6.2 Yeast splicing factors

In yeast, the ability to isolate splicing mutants has allowed the identification of a number of genes encoding putative splicing factors. An early study by Hartwell (1967) isolated 400 temperative sensitive mutants which were defective in macromolecule synthesis. Of these, 23, falling into 9 complementation groups (prp2 to prp 10/11, formerly rna2 to rna 10/11) ceased synthesis of RNA at the restrictive temperature. These have subsequently been shown to primarily affect RNA splicing (Rosbash et al, 1981). Since most ribosomal protein genes contain introns, a block in RNA splicing inhibits synthesis of these proteins, which in turn rapidly inhibits the synthesis of rRNA. rRNA comprises the majority of cellular RNA, so an indirect effect of the block in pre-mRNA splicing is a rapid cessation of synthesis of the majority of cellular RNA. In a recent study 11 further complementation groups were isolated (Vijayraghavan et al, 1989). Among these were mutants in which only the second step of splicing was inhibited. It has been shown that in vitro splicing extracts made from all the prp strains, with the exception of prp6 and prp9, are temperature

sensitive *in vitro*, suggesting that these gene products are directly involved in splicing (Lustig *et al*, 1986).

Several of the *PRP* genes have been cloned by complementation of the temperature sensitive phenotype (Lee *et al*, 1984; Last *et al*, 1984; Soltyk *et al*, 1984; Jackson *et al*, 1988). The gene products (Chang *et al*, 1988; Banroques and Abelson, 1989) or fusion proteins derived from these genes (Lee *et al*, 1986; Last and Woolford, 1986; Peterson-Bjorn *et al*, 1989; Jackson *et al*, 1988; this thesis) have been expressed in *E coli* and used to raise antisera to the proteins. This has in turn allowed identification of the gene products in yeast, and partial elucidation of their role in RNA splicing. Thus, PRP8 protein has been shown to be associated with the U5 snRNP (Lossky *et al*, 1987) and also to bind directly to the pre-mRNA during RNA splicing (E. Whittaker, personal communication). PRP4 protein is associated with the U4/6 snRNP (Peterson-Bjorn *et al*, 1989; Banroques and Abelson, 1989). By synthesising radio-labelled PRP11 protein in an *in vitro* translation system, it was shown that this protein is associated with splicing complexes (see section 1.7.2), but not with any individual snRNP (Chang *et al*, 1988).

The *PRP2* gene has been cloned (Lee *et al*, 1984; Last *et al*, 1984) and its sequence determined (J. Beggs, unpublished). This sequence has revealed two interesting homologies. The first is the presence of the consensus sequence for the 'zinc finger' structure which is present in a number of nucleic acid binding proteins . The other major homology is to the enzyme adenylate kinase, which binds ATP, although the sequence found in PRP2 protein contains only the part of the ATP binding site which is responsible for phosphate group binding. The significance of these homologies for the function of PRP2 protein is uncertain.

PRP2 protein has been identified by immunological methods and has a molecular weight of approximately 100kD, by SDS-PAGE (Lee *et al*, 1986; Last and Woolford, 1986). Immunofluoresence studies using anti-PRP2 antibodies have shown that the protein is localized in the cell nucleus (Last and Woolford, 1986). PRP2 protein appears to be distinct from the other *PRP* gene products in that when prp2 splicing extracts are heat inactivated,

splicing complexes form (Lin et al, 1987). In this study another factor, b_n, which appears to be required for the activity of PRP2 protein, was detected.

Two other factors which are required for step 2 of the splicing reaction have been identified by biochemical fractionation (Cheng and Abelson, 1986).

Another approach to the identification of trans-acting factors is the isolation of extragenic suppressors of already existing mutations, of either the pre-mRNA or of the prp genes. Using the first approach, prp16 a gene encoding a protein which suppresses the branchpoint sequence mutation UACUACC was isolated (Couto et al, 1987) . This gene is distinct from the U2 snRNA gene.

Using the second approach, cold sensitive suppressors of prp2 and prp8 have been isolated in this laboratory (D.Jamieson, unpublished). A suppressor of prp2 was isolated, which itself is required for splicing (Last et al, 1987): this protein might be the b_n factor (Lin et al, 1987). It has also been shown that high levels of PRP3 protein suppress the prp4 defect and that two prp4 suppressors also suppress prp3 (Last et al, 1987). Given that PRP4 protein is associated with U4/6 snRNP, it is probable that PRP3 protein is also associated, either directly or indirectly, with this snRNP.

1.6.3 Schizosaccharomyces pombe splicing factors

Introns are more common in Schizosaccharomyces pombe than in S. cerevisiae, and the SV40 large T intron is spliced in this organism (Kaufer et al, 1985), although other mammalian introns tested are not spliced. Three temperature sensitive pre-mRNA splicing mutant strains were recently isolated (Potashkin et al, 1989), but as yet no in vitro splicing system has been described for this organism.

1.7 Splicing Complexes

1.7.1 Mammalian splicing complexes

As might be expected from the fact that pre-mRNA splicing requires the binding of four snRNP particles, as well as a number of other factors, to the RNA, pre-mRNA splicing takes place in a large complex. This complex, termed the spliceosome was first observed on sucrose gradients as a 50-60S peak of radioactivity (Frendewey and Keller, 1985; Grabowski et al, 1985). It was shown that this peak contained splicing intermediates, in addition to pre-mRNA. When pooled peak fractions were mixed with splicing extract, the pre-mRNA was converted to intermediates and products without the time lag which normally occurs before initiation of in vitro splicing (Grabowski et al, 1985). Spliceosomes have now been observed and purified by non-denaturing gel electrophoresis (Konarska and Sharp, 1986; Lamond et al, 1987), affinity chromatography (Grabowski and Sharp, 1986; Bindereif and Green, 1987) and gel filtration followed by electron microscopy (Reed et al, 1988).

The initial experiments with sucrose gradients showed that in the lag period before splicing commences, a 35-40S complex forms, which then is converted to the full size 50S-60S spliceosome (Frendewey and Keller, 1985; Grabowski et al, 1985). At the same early times a complex appears on non-denaturing gels, which is referred to as complex A; at later time points a more slowly migrating complex, B, forms, which contains splicing intermediates (Konarska and Sharp, 1986). It is assumed that complex A corresponds to the 35-40S peak and complex B to the 50-60S peak.

Formation of complex A requires ATP (Konarska and Sharp, 1986) and the presence of a polypyrimidine tract in the substrate RNA (Frendewey and Keller, 1985; Bindereif and Green, 1986) . In the absence of a 5' splice site, a complex forms which has the same apparent sedimentation coefficient as the 35-40S complex on sucrose gradients (Frendewey and Keller, 1985; Konarska and Sharp, 1986), but under appropriate conditions this complex can be distinguished from the authentic complex A on non-denaturing gels (Zillmann et al, 1988). Initially, it was not possible to detect U1 snRNP

in spliceosomes (Konarska and Sharp, 1986; Lamond et al, 1987; Grabowski and Sharp, 1986), which was unexpected, given its established role in 5' splice site binding, and the fact that anti-U1-RNP antibodies co-precipitate splicing intermediates (Grabowski et al, 1985). This was later shown to be due to the treatment of splicing reactions with heparin and high salt, in order to remove non-specific RNA binding proteins from the pre-mRNA by competition. Under less stringent conditions it was possible to detect U1 in spliceosomes (Bindereif and Green, 1987; Reed et al, 1988; Zillmann et al, 1988).

Analysis of the kinetics of snRNP binding suggests that U1 snRNP binds to the substrate first, followed by U2 snRNP (Bindereif and Green, 1987). Several pieces of data suggest that the initial binding of U1 snRNP is not to the 5' splice site, but requires the presence of 3' splice site sequences. Thus, U1 snRNP is required for formation of the A-like complex on substrates lacking a 5' splice site (Zillmann et al, 1987). Furthermore, cleavage of the 5' end of U1 RNA with oligonucleotides plus RNase H inhibits, but does not abolish co-precipitation of substrate RNA with anti-U1-RNP antibodies, and this binding depends on the presence of 3' splice sequences, but not on 5' splice site sequences (Zillmann et al, 1987). As mentione din section 1.6.1, in the absence of a specific factor U1 snRNP binds to the pre-mRNA, but does not protect the 5' splice site (Zapp and Berget, 1989). However, immunodepletion or oligonucleotide-directed cleavage of U1 snRNP inhibits the binding of the other snRNPs, suggesting a 'hierarchic' role of U1 snRNP in splicing complex assembly (Zillmann et al, 1987).

Cleavage of the 5' end of U2 RNA inhibits formation of complex A (Zillmann et al, 1988; Frendewey et al, 1987; Chabot and Steitz, 1987a), while cleavage of the U2 RNA sequence which is capable of base pairing with the branchpoint sequence, did not inhibit complex A formation (Zillmann et al, 1988). Another study showed that branchpoint mutations which decrease the degree of complementarity to U2 RNA did not inhibit spliceosome formation (Reed and Maniatis, 1988). These results suggest that although U2 snRNP protects the branchpoint region from digestion with T1 RNase, at early stages in complex assembly, it is not base paired to this sequence.

However, other data (Lamond et al, 1989) have suggested that U2 snRNP is base paired to the branchpoint region at early stages of spliceosome assembly. At later stages it is clear that base pairing between U2 RNA and branchpoint sequences is important (see section 1.5.3.1). The association of U2 snRNP with the RNA requires ATP and 3' splice site sequences, which are bound by the U2 snRNP activating factor (U2AF, section 1.6.1).

In the transition to complex B, the U4/6 and U5 snRNPs bind simultaneously (Konarska and Sharp, 1986). The formation of an ATP-dependent complex between U5 and U4/6 snRNPs, prior to incorporation into spliceosomes has been detected (Konarska and Sharp, 1987). Under conditions in which complex B could be resolved into two complexes, U4 RNA was found to be absent from the more slowly migrating complex, which contained the splicing intermediates (Lamond et al, 1988). However, it is possible that this result is due to instability of the interaction of U4 snRNP with the spliceosome under gel electrophoresis conditions. It has recently been demonstrated, using an oligonucleotide complementary to U4 that it is possible to detect U4 snRNP in complexes containing the lariat intron product (Blencowe et al, 1989), suggesting that U4 snRNP is present in spliceosomal complexes throughout the reaction. There must, however, be a weakening of the interactions between U4 snRNP and the spliceosome, immediately prior to formation of the active spliceosome.

In the 50-60S spliceosome the size of the 5' splice site fragments protected by U1 snRNP increases (Chabot and Steitz, 1987b). Following step 1 of the reaction, the U2 snRNP appears to move slightly downstream (Kramer, 1987), while fragments of exon 1 become protected from RNase T1 digestion by U1 snRNP (Chabot and Steitz, 1987b). In this study, protection of both 5' splice site and branchpoint fragments by both U1 snRNP and U2 snRNP was observed, indicating that some interactions between these snRNPs must occur.

When the splicing reaction is completed, spliceosomes dissociate. The lariat intron product is released in a complex with at least the U2, 5 and 6 snRNPs (Konarska and Sharp, 1987), while mRNA is found in complexes

similar to those which form on pre-mRNAs prior to assembly into spliceosomes.

1.7.2 Yeast splicing complexes

Yeast spliceosomes were initially observed as 40S peaks on glycerol gradients (Brody and Abelson, 1985). As in the mammalian case, they contained splicing intermediates and required ATP for their formation. In general, mutations which block step 1 of the yeast splicing reaction strongly inhibit spliceosome formation. A study of chemical modification of pre-mRNAs revealed the dependence of different steps of spliceosome assembly, and of the splicing reaction, upon different nucleotides, (including those immediately adjacent to the consensus sequences) (Rymond and Rosbash, 1988) .

The first event detected in yeast spliceosome assembly is the binding of U1 snRNP to form a complex which is 'committed' to splicing (Seraphin and Rosbash, 1989). Efficient formation of this complex requires both an intact 5' splice site and branchpoint sequence. Cleavage of U1 RNA inhibits association of other snRNPs with the pre-mRNA (Ruby and Abelson, 1988). The second complex to form contains U1 and U2 snRNPs and has been referred to as complex B (Cheng and Abelson, 1987), or complex III (Pikielny et al, 1986). As noted in section 1.5.3.2., the 5' terminal region of U1 RNA interacts with the 5' splice site through Watson-Crick base pairing in spliceosomes (Seraphin et al, 1988; Siliciano and Guthrie, 1988). The binding of U2 snRNP to the pre-mRNA requires the presence of U1 snRNP at the 5' splice site, but may also depend to some degree on the 5' splice site sequences themselves (Ruby and Abelson, 1988). It has been shown that U2 RNA is base paired to the UACUAAC sequence (Parker et al, 1987), in spliceosomes.

Following the formation of complex B (III) there exists a difference between the observed patterns of spliceosome formation. Cheng and Abelson (1987) observed three further, more slowly migrating complexes, which appeared in the order A2-1, A1, A2-2. A2-1 and A2-2 co-migrate in the gel system used by these investigators while A1 migrates more slowly than

these two and appears only transiently. Only A2-2 contained splicing intermediates, and was therefore defined as the active spliceosome. Pikielny et al (1986) observed two complexes, I and II (numbered in order of increasing electrophoretic mobility), migrating more slowly than complex III . The order of appearance of complexes was III, I, II, with II being the active spliceosome. Investigation of the snRNP composition of these complexes revealed that complex A2-1 contained U2, U4, U5 and U6 RNAs (U1 was not detected in these experiments, cf section 1.7.1), while A1 and A2-2 contained only U2, U5 and U6 RNAs. Complex I contained U2, U4, U5 and U6 RNAs while complex II contained U2, U5 and U6. Whatever the relationship between complex A2-1 , A1 and I, as in mammalian systems, U4 RNA appears to dissociate from spliceosomal complexes immediately prior to formation of the active spliceosome. (It should be noted, however, that this may only reflect a change in U4 association with spliceosomes, which makes it unstable to gel electrophoresis conditions, cf. section 1.7.1).

The 3' splice site is not required for step 1 of the reaction, and as expected, active spliceosomes form upon substates lacking a 3' splice site (Rymond et al, 1987). However, for the RP51A gene, approximately 29 nucleotides downstream of the UACUAAC sequences are necessary for step 1, and pre-mRNAs with less than this length of RNA sequence 3' to the UACUAAC sequence do not undergo the transition from complex I to complex II. It is interesting to note that complexes formed on such substrates do contain U5 RNA despite the fact that, in mammalian systems, U5 snRNP is thought to be associated with the 3' splice site region (section 1.5.2).

During the transition from complex III to complexes I and II, the degree of protection of the 5' splice site from oligonucleotide-directed RNaseH cleavage increases (Rymond and Rosbash, 1986). This finding is similar to results in mammalian systems (Chabot and Steitz, 1987b). In yeast spliceosomes, only the 5' splice site and UACUAAC sequence are protected from oligonucleotide-directed RNaseH cleavage.

1.8 Splice Site Selection and Alternative Splicing

In mammalian systems, pre-mRNAs generally contain sequences which have a good match to the splice site consensus sequences, but which are not used in splicing. Studies in which competition between 5' splice sites was examined (Eperon *et al* 1986, Nelson and Green 1988) indicated that the consensus 5' splice site is the most effective competitor, and in general, the 5' splice which is used has greater complementarity to U1 RNA than any possible cryptic splice site. It was also shown that increasing the complementarity of the SV40 large T 5' splice site to U1 RNA improved its efficiency of usage (Yuan *et al* 1987). However Nelson and Green (1988), demonstrated that the sequence context of splice sites also has complex effects on efficiency of 5' splice site usage. Their data suggested that regions in the proximity of the authentic 5' splice site are more 'permissive' for splicing than other regions of the transcript. Reed and Maniatis (1986) and Cooper and Ordahl (1989) also found effects of exon sequences on 5' splice site usage. Where a number of 5' splice sites compete for a single 3' splice site, the most upstream site is often preferred (Kedes and Steitz, 1986).

Although earlier studies indicated that mutation of branchpoint sequences had little effect on splicing efficiency, recent investigations, using cis-competition assays have revealed that splicing efficiency can be affected by the match of the sequence around the branchpoint to the consensus, (and thereby, complementarity to U2 snRNP; Zhuang *et al* 1989; Reed and Maniatis, 1988). In the case of the SV40 T/t intron, branch site mutations can effect the relative efficiency of usage of the two 5' splice sites *in vivo* (Noble *et al*, 1988).

As these results make clear, no simple scanning mechanism (5' to 3' or 3' to 5') can account for the choice of pairs of splice sites, although some element of scanning cannot be excluded. The mechanism may differ for different pre-mRNAs, and probably depends upon non-conserved sequence elements, in addition to high order pre-mRNA structure effects.

An increasing number of cases are being discovered of alternative patterns of splicing of a single primary transcript (reviewed in Breitbart et al, 1987). This phenomenon underlies the production of different isoforms of many proteins, often in a cell type or developmental stage-specific pattern. There are several types of alternative splicing: (i) omission or retention of a single exon; (ii) the retention or omission of a single intron; (iii) mutually exclusive splicing of two exons; (iv) use of alternative 5' splice sites with a single 3' splice site, or vice versa.

An example of the first of these types is the rat fast skeletal troponin T gene, in which any of exons 4 to 8 may be retained or omitted from the mRNA, independently of each other, to give 32 possible isoforms (Breitbart et al, 1985). This process is regulated in a tissue and developmental stage specific manner.

Two examples of retention/omission of an intron occur in D.melanogaster: the auto regulated splicing of the su(w^a) pre-mRNA (Zachar et al 1987; Chou et al, 1987), and the retention of the third intron in the P-element primary transcript in somatic cells, but not in the germ line (Laski et al, 1986). In the former case, as might be expected, the retained introns contain translation termination codons, resulting in the production of non-functional mRNAs. In the rat γ-fibrinogen gene, on the other hand, the open reading frame is maintained throughout the retained intron (Crabtree and Kant, 1982; Chung and Davie, 1984).

The mutually exclusive inclusion of two exons in an mRNA has been studied in the rat α-tropomyosin gene (Wieczorek et al, 1988) in which the choice between three pairs of exons determines whether the smooth, striated muscle, or nonmuscle isoforms of the protein is produced. Where mutually exclusive exons are present in a pre-mRNA, the intron between them is a 'pseudo intron', since it is never spliced. An example is the intron between α-tropomyosin exons three and four, which is not spliced due to the short distance between the 5' splice site and branchpoint sequence (Smith et al, 1989).

The earliest discovered examples of alternative splicing, the T/t splice in SV40, involves use of alternative 5' splice sites and a common 3' splice site (Reddy et al, 1978).

One of the best understood example of regulated splicing is the cascade of alternative splicing events in D. melanogaster somatic sex determination (Baker, 1989). The initial signal, the ratio of X chromosomes to autosomes, gives rise to the activity of the sxl gene in females only, due to autoregulation of sxl splicing (ommission of an exon which contains a stop codon) by the sxl protein. In females, the sxl protein also partially blocks the (default) male-specific splicing of the tra pre-mRNA, resulting in production of active tra mRNA, which also lacks an exon containing a stop codon. In females, the tra protein, in concert with the tra-2 protein, directs female specific splicing (inclusion of exon 4, rather than exons 5 and 6) of the dsx pre-mRNA. The male and female specific forms of the dsx protein control expression of genes which cause male or female somatic differentiation.

In yeast, the only case of regulated splicing so far discovered is the auto-inhibition of splicing of the RPL32 gene (Dabeva et al, 1986). Other ribosomal protein genes which were tested do not exhibit a similar auto-regulation.

Clues to the mechanism of regulation of alternative splicing by the sxl, tra and tra-2 proteins are the presence in all these proteins of the 80 amino acid consensus sequence common to a number of RNA binding proteins (Dreyfuss, 1989). In addition, tra and tra-2, possess an arginine and serine rich domain, also found in U1 70K protein and in the su(w^a) protein, which also regulates the splicing of its own pre-mRNA (Bandziulis et al, 1989).

Other candidate trans-acting factors, which may be responsible for tissue and developmental stage specific regulation of splicing, include the different forms of U1 and U4 RNA (Forbes et al, 1984; Lund et al, 1985; Lund et al; 1987, Lund and Dahlberg, 1987; Korf et al, 1988). Expression of in different tissues of N , a protein related to the B and B' snRNP proteins, appears to be correlated with the ability of cells of that tissue

to perform the splice which produces CGRP (calcitonin-gene related peptide) instead of calcitonin (MacAllister *et al*, 1988, 1989). As yet, *in vitro* systems which reproduce the patterns of alternative splicing found *in vivo* have not been described.

1.9 Trans-splicing

All the results discussed above concern splicing of exons which are encoded within a single pre-mRNA. It has recently been discovered, however, that splicing of exons on different pre-mRNAs can also occur. The initial finding was that all pre-mRNAs in *Trypanosoma brucei* contained a common 35 nucleotide leader sequence at their 5' ends (van der Ploeg, 1986). This segment of RNA derives from a 137 nucleotide RNA, which is transcribed from multiple clusters of repeated DNA sequences. The 35 nucleotide leader is found at the 5' end of the 137 nucleotide RNA, and is followed by a consensus 5' splice site, while the bodies of *T. brucei* mRNAs have a 3' splice site consensus sequence near their 5' ends. It was shown thatthe 5' leader is joined to the body of the pre-mRNA by a process of trans-splicing (Murphy *et al*, 1986; Sutton and Boothroyd, 1986). Trans-splicing has also been found in *Caenorhabditis elegans* (Blumenthal and Thomas, 1988) and in other nematodes (Bektesh *et al*, 1988).

The 5' and 3' splice sites sequences in trans-splicing conform to the consensus sequences, and branched intermediates have been detected (Murphy *et al* 1986; Sutton and Boothroyd, 1986). The branchpoint residues are adenosines, located 44 and 56 nucleotides upstream from the 3' splice site in the *T. brucei* α- and β-tubulin genes, but the branchpoint sequences have no homology to the mammalian branchpoint consensus sequence (Patzelt *et al* 1989). However, there is a sequence in *T. brucei* U2 RNA, from nucleotides 48-58, which can potentially base pair with all the observed branchpoint sequences.

It appears that the 137 nucleotide RNA performs the function of U1 snRNP in trans-splicing: it has an m_3G cap structure, a 'domain A' Sm-binding site and is Sm-precipitable (Thomas *et al*, 1988; van Doren and

Hirsch, 1988; Bruzik et al, 1988). In fact, T. brucei has no detectable U1 homologue (Mottram et al, 1988).

There is no homology between the 137 nucleotide RNA and the bodies of T. brucei mRNAs, so it is likely that the two halves of the trans-spliceosome form independently and then recognise each other. Such a mechanism has been observed in mammalian in vitro splicing extracts (Konarska et al 1985b).

1.10 The Evolution of RNA Splicing

The main question that arises with reference to the evolution of RNA splicing is that of the antiquity of introns. With a number of exceptions, prokaryotic genes do not contain introns, whereas they are widespread in eukaryotic genes (S. cerevisiae being an intermediate case). It is possible to envisage either that introns pre-date the divergence of prokaryotes and eukaryotes and have been subsequently eliminated from the former (Doolittle, 1978; Gilbert, 1978, 1985), or that they were absent in ancestral genes, and have become inserted into eukaryotic genes at a later stage (Orgel and Crick, 1980).

A number of observations favour the former hypothesis. Firstly, the existence of introns in some prokaryotic genes (Schmidt, 1985) and in chloroplasts (Shih et al, 1988) suggest that these introns were present in the genes of the common ancestor of prokaryotes and eukaryotes. Secondly, the structure of many intron-containing genes suggests that during evolution they have lost introns by precise excision (Perler et al, 1980; Shah et al, 1982; Cooper and Crain, 1982). An extension of this hypothesis is the idea of 'genes-in-pieces', i.e. that exons code for domains, or modules (Go, 1981; 1983) of protein structure, which can be assembled in different combinations to form different proteins. The separation of exons by introns would allow recombination within the introns, resulting in 'exon shuffling', which would generate evolutionary diversity more rapidly than point mutation within exons. The structure of many genes, in particular the immunoglobulin/histocomp-atability antigen/T cell receptor

family (Honjo, 1983) supports the idea that exons correspond to protein domains. The correspondence of exons with protein structure modules is evident in the glycolytic enzymes pyruvate kinase (Lonberg and Gilbert, 1985) and triosephosphate isomerase (Straus and Gilbert, 1986). Analysis of the structure of the LDL receptor gene (Sudhof et al, 1985a, b) suggests strongly that it has arisen by processes of intron loss and exon shuffling. It is, however, also clear that certain introns in eukaryotes and prokaryotes are mobile, and capable of inserting by DNA recombination into genes which contain the correct target sequence (Lambowitz, 1989). It therefore appears that processes of both intron insertion and elimination have contributed to the pattern of introns in present day genes.

In prokaryotes the vast majority of genes do not contain introns. The hypothesis of Doolittle (1978) suggests that in these organisms there has been a higher selective pressure in favour of intron loss, due to the need to minimize genome size, in order to allow rapid DNA replication.

One problem with the idea of introns in ancestral genes is the necessity for a machinery for accurate and efficient RNA splicing. However, the discovery that some group I and group II introns can catalyse their own excision in vitro (Cech and Bass, 1986), in the absence of proteins, has provided a possible resolution to this problem.

The mechanism of the splicing reaction for group I, II and pre-mRNA introns is similar, having two steps. In the first step, the 5' splice site is cleaved by a nucleophilic attack, of a free guanosine nucleotide for group I introns, or of a 2' hydroxyl group of a residue near the 3' end of the intron, for group II and pre-mRNA introns. In the second step of the reactions, the 3' hydroxyl group of exon 1 makes a nucleophilic attack on the phosphodiester bond at the 3' splice site, producing spliced RNA and the excised intron. These similarities in mechanism suggest that the three different groups of introns have a common ancestor, which was probably a self-splicing intron. Thus, the RNA molecules in the common ancestor may have played both informational and enzymatic roles (Gilbert, 1986): the Tetrahymena thermophila rRNA group I intron is also an oligo-C polymerase (Zaug and Cech, 1986), suggesting that RNA could catalyse its

own replication (Cech, 1987). At later stages of evolution, most of the catalytic functions of RNA have been taken over by proteins, whereas the long term information storage role has been taken over by DNA. In the case of group I and II RNA splicing, the RNA catalytic role has been retained, although, *in vivo*, proteins are required to stabilize the conserved RNA secondary structures in the intron, which are necessary for splicing. This suggests that the catalytic molecules in nuclear pre-mRNA splicing may be the U snRNAs, which may have evolved to replace the conserved secondary structure elements of group I and II introns. It is, however, possible that the catalytic molecule in pre-mRNA splicing is a protein.

1.11 This Thesis

In this thesis I present the following:

(i) production and characterization of antibodies to β-galactosidase-PRP2 fusion proteins;

(ii) construction of systems for over-expression of PRP2 protein, in *S. cerevisiae*, *E. coli* and *in vitro*;

(iii) development of an assay system for PRP2 protein activity, based on a system described by (Lustig *et al*, 1986);

(iv) partial purification of PRP2 protein;

(v) analysis of interactions between PRP2 proteins and yeast spliceosomes, including (a) demonstration of direct association of PRP2 protein with spliceosomes, and (b) failure of heat-inactivated prp2 protein to associate with spliceosomes, (c) analysis ofinteraction of PRP2 protein with specific spliceosomal complexes and (d) mapping of the PRP2 protein binding site relative to pre-mRNA, in spliceosomes.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Suppliers of laboratory reagents

Restriction enzymes and other DNA modifying enzymes: Amersham International, Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs and Pharmacia.

Radiochemicals: Amersham International, New England Nuclear.

Deoxyribonucleotides and dideoxyribonucleotides: Pharmacia.

Ribonucleotides: Sigma and Pharmacia.

Acrylamide and NN'-methylene bisacrylamide: BDH Chemicals, "Electran" grade.

Agarose, Ultra-Pure Agarose and Low Melting-Point Agarose: Sea-Kem and BRL.

Media reagents: Difco Labs.

Standard laboratory reagents (analytical grade, or better): BDH, Fisons, Sigma, May and Baker, Koch-Light, Bio-Rad and Serva.

2.1.2 Growth media

All quantities listed are for 1 litre, unless otherwise stated. Solutions were autoclaved (see section 2.2.2) and stored at room temperature. Antibiotic and amino acid stock solutions were added to media immediately before use.

2.1.2.1 E. coli media

Luria broth (LB):	10g Bacto-Tryptone , 5g yeast extract , 10g NaCl, adjusted to pH7.2 with NaOH.
Luria-agar (LB-agar):	LB plus 15g agar.
LB/ampicillin:	LB plus 0.1mg/ml ampicillin.
M9+CA medium:	6g $Na_2HPO_4.H_2O$, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl, 5g casamino acids , 2g glucose, 10mg vitamin B1, 0.25g $Mg_2SO_4.7H_2O$, 22mg $CaCl_2.7H_2O$.
M9+CA+W medium:	M9+CA medium plus 20mg tryptophan.
Ampicillin stock:	0.1g/ml, stored at -20°C.

2.1.2.2 Yeast media

YPDA:	10g Bacto-Tryptone, 20g Bacto-Peptone, 20g glucose, 20mg adenine sulphate.
YPDA-agar:	YPDA plus 20g agar .
YPDAS:	YPDA plus 218g sorbitol.
YMM:	6.7g yeast nitrogen base (without amino acids), 20g glucose (supplemented with amino acids to 40µg/ml as required).
YMM-agar:	YMM plus 20g agar .
YMMS:	YMM plus 218g sorbitol .

YMMS bottom agar: YMMS plus 20g Bacto-Agar.

YMMS top agar: YMMS plus 30g Bacto-Agar.

Amino acid stocks: 4mg/ml in dH_2O; filter sterilised and stored at 4°C.

Uracil: 2mg/ml in dH_2O; filter sterilised and stored at 4°C.

2.1.3 Bacterial strains

All strains were derivatives of *E. coli* K-12.

TABLE 2.1 Bacterial Strains

Strain	Genotype	Reference
HB101	F, *hsdS20*(r^-B, m^-B), *recA13 ara-14*, *proA2*, *lacY1*, *galK rpsL20*(Sm^r), *xyl-5*, *mtl-1*, *supE44*.	Boyer and Roulland-Dussoix, (1969)
DH5	F^-, *recA1*, *endA1* *gyrA96*, *thiI*, *hsdR17* (r^-K, m^+K), *supE44*.	Hanahan *et al* (1985)
BMH71-18	Δ(*lac*, *pro*), *thi*, *supE*, F', (*lacI*^{q}, *lacZ*ΔM15, *pro*+)	Messing *et al* (1977)

2.1.4 Yeast strains

TABLE 2.2 Yeast Strains

Strain	Genotype	Source
BJ2412	a/α, ura3-52/ura3-52, leu2/leu2, trp1/trp1, gal2/gal2, pep4-3/pep4-3, prb1-1122/prb1-1122, prc1-407/prc1-407	E. Jones (Pittsburgh)
KY117	a, his3-Δ200, lys2-801^{am}, ade2-101, trp1-Δ1, ura3-52, GAL^{+}	K. Struhl (Harvard)
KY118	α, his3-Δ200, lys2-801^{am}, ade2-101, trp1-Δ1, ura3-52, GAL^{+}	K. Struhl (Harvard)
DBY747	a, his3-Δ1 leu2-3, leu2-112 trp1-289 ura3-52, gal^{-}	D. Botstein (MIT)
DJY40	a, his7, ura3, lys2, leu2 GAL^{+}	D. Jamieson (this lab)
JBY27	a, prp2-1, leu2-3 leu2-112, his3-Δ200, trp1, ura3, lys2-801^{am}, ade1/2, gal	J. Beggs (this lab)
DJY39	α, prp2-4, ura3-52 his3, his7, leu2 tyr1, ade1/2, gal^{-}	D. Jamieson (this lab)

TABLE 2.2 (cont.) Yeast Strains

Strain	Genotype	Source
DJY85	a/α, prp2-1/prp2-1, ura3-1,2/ura3, ade1/ADE1, ade2/ade2, HIS3/his3-Δ200, tyr1/TYR1, LYS2/lys2-801^{am}, TRP1/trp1, can1/CAN1	D. Jamieson (this lab)

2.1.5 Plasmid vectors and constructs

Table 2.3 Plasmid Vectors and Constucts

Plasmid	Description	Source	Reference
pUR278 pUR288 pUR289 pUR290 pUR291 pUR292	*E. coli* plasmids; multiple cloning sites in 3' end of lacZ gene, amp^R. For synthesis of IPTG-inducible β-galactosidase fusion proteins, in strain BMH71-18.	D. Lane (ICRF, Clare Hall)	Ruther and Muller-Hill (1983)
pATH-1 pATH-2 pATH-3	*E. coli* plasmid; multiple cloning sites in 3' end of *trpE* gene, amp^R. For synthesis of IAA-inducible trpE fusion proteins.	D. Finnegan (Edinburgh)	Dieckmann and Tzagaloff (1985)
pDR540	*E. coli* plasmid; tac promoter followed by BamHI site, amp^R. For IPTG-inducible expression of proteins.	Pharmacia	
pSPT18	*E. coli* plasmid; multiple cloning site flanked by bacteriophage T7, SP6 promoters, amp^R. For *in vitro* RNA synthesis.	Pharmacia	

Table 2.3 (continued)

Plasmid	Description	Source	Reference
pFP2.1	pUR290 with 460bp Sau3A fragment of PRP2 gene.	M. Lee (this lab)	Lee et al, 1986
pJDB207	Yeast E.coli shuttle plasmid; high copy number, LEU2; amp^R	J. Beggs (this lab.)	Beggs (1981)
pBM125	Yeast E.coli shuttle plasmid, low copy number, URA3, CENIV. Contains yeast GALI UAS and promoter. amp^R	M. Johnston, R. Davis (Stanford)	
pKV49	Yeast E.coli shuttle plasmid; high copy number, PGK promoter 3' to GALI UAS, LEU2. BglI cloning site at 3' end of promoter. amp^R.	D . Cousins (Oxford)	
pY2000	YEp24 with 7.7kb Sau3A insert containing PRP2 gene.	M. Lee (this lab)	Lee et al (1984)
pY2519+	YCp50 with PRP2 gene and HIS3 gene inserted at BamHI site, 3' to PRP2.	M. Lee (this lab)	

Table 2.3 (continued)

Plasmid	Description	Source	Reference
pY2076	pJDB207 with 3.2kb EcoRI-BamHI insert containing PRP2 gene.	M. Lee (this lab)	Lee et al (1984)
pSPRP51ApA	pSP62 with yeast RP51A gene (with region encoding polyA tail), downstream of SP6 promoter.	C. Pikielny (Brandeis)	Pikielny et al (1986)
pSPRP51AΔ2	Derivative of pSPRP51ApA, with 263nt deletion from RP51A intron.	C. Pikielny (Brandeis)	Pikielny et al (1986)
pSPRP51AΔ3B	Derivative of pSPRP51ApA 37nt deletion from RP51A in intron, covering TACTAAC. sequence.	C. Pikielny (Brandeis)	Pikielny et al (1986)
pSPTact/alu	pSPT19 carrying 544 bp AluI fragment of ACT1 gene.	M. Lossky-Elias (this lab)	Jackson et al (1988)

2.1.6. Miscellaneous materials

2.1.6.1 Antisera

Antibodies (purified by ammonium sulphate precipitation) against the m_3G cap structure were a gift from R. Luhrmann (Marburg).

Human Sm antiserum (Kung) was provided by I. Mattaj (Heidelberg).

Rabbit anti-PRP8 antiserum (αFP8.4, rabbit 58) was provided by G. Anderson (this lab).

2.1.6.2 DNA oligonucleotides

Yeast RP51A	: 5' - TAAAAAGTTTGTAAATGCAA - 3'
Yeast RP51A/C	: 5' - AAATGACGAAAAGCAATACA - 3'
Yeast RP51A	: 5' - AATATCGTCATATGTTTTCT - 3'
Yeast U1	: 5' - CTTAAGGTAAGTAT - 3'
Yeast U2	: 5' - CTACACTTGATCTAAGCAAAAGGC - 3'
Yeast U4	: 5' - TTTCAACCACGAAA - 3'
Yeast U5	: 5' - AATATGGCAAGCCC - 3'
Yeast U6	: 5' - TC(T/A)TCTCTGTATTG - 3'

DNA oligonucleotides were custom synthesised by the OSWEL DNA service (Dept. of Chemistry, Edinburgh University), except the oligonucleotide complementary to yeast U6 RNA which was a gift from D. Brow (UCSF), and that complementary to yeast U2 RNA, which was a gift from D.Field (Toronto)

2.1.6.3 Others

AflIII was a gift from N. Brown (Birmingham). Control RNA for in vitro translation (a mouse DHFR transcript made by in vitro transcription) was kindly provided by G. Reid.

2.2 General Methods

2.2.1 General procedures

Unless stated otherwise, all procedures involving liquid volumes of less than 1.5ml were carried out in sterile, capped, 1.5ml polypropylene tubes. Larger scale procedures were performed in 10ml or 50ml sterile Falcon tubes, in washed, non-sterile, 15ml or 30ml glass Corex tubes and in other unsterilized laboratory glassware (conical flasks, beakers, etc.). Liquids were dispensed using Gilson Pipetman P-20, P-200 and P-1000 automatic pipettors and sterile plastic tips for volumes less than 1ml, and with sterile glass pipettes for volumes greater than 1ml. All procedures were performed at room temperatures, unless otherwise stated.

Centrifugation of volumes less than 1.5ml was performed in an Eppendorf microcentrifuge at 13,000 rpm (13,800g). Volumes of up to 50ml were centrifuged in an 1ED Centra-4X bench top centrifuge with a swing-out rotor, an MSE Minor'S' bench top centrifuge with a fixed angle rotor, or a Sorvall RC-5B centrifuge with HB-4 swing out rotor or SS34 fixed angle rotor. Larger volumes were centrifuged in a Sorvall RC-5B centrifuge, using GSA and GS-3 rotors. Ultracentrifugation was performed in Sorvall OTD50B or OTD55B ultracentrifuges, using Ti50 rotors.

2.2.2 Preparation and storage of solutions

All solutions were prepared using distilled or double-distilled water. Solutions were stored at room temperature unless otherwise stated. Enzymes, nucleotide solutions and reaction buffers were stored at -20°C or -70°C.

2.2.3 Sterilization of solutions

Stock solutions were sterilized by autoclaving at 15psi, at 120°C for 20 minutes, or by filtration through Acrodisc filters (0.45μm pore size; Gelman Sciences) in the case of solutions which were unstable on heating.

2.2.4 Deionization of solutions

Solutions were deionized where necessary by stirring, for 30 minutes at room temperature with approximately 0.1 volume of mixed-bed ion-exchange resin (20-50 mesh, Bio-Rad laboratories). The resin was removed by filtration through Whatman no.1 filter paper.

2.2.5 Autoradiography

^{32}P-labelled nucleic acids, in gels or on filters, were detected by exposure of X-ray film (Du Pont Cronex, or Amersham Hyperfilm-MP) in light-proof, lead-shielded cassettes. The method of exposure depended upon the sensitivity desired. In order of increasing sensitivity, these were: (i) at room temperature; (ii) at -70°C with a calcium-tungstate intensifying screen; (iii) at -70°C with a pre-flashed film and calcium-tungstate intensifying screen (Laskey, 1984).

^{35}S-labelled proteins, in a gel soaked in Amplify (Amersham) were detected by exposure of pre-flashed X-ray film (Du Pont Cronex) at -70°C.

^{125}I-labelled proteins on filters, were detected using pre-flashed X-ray film (Du Pont Cronex) at -70°C.

2.2.6 Dialysis

Dialysis tubing (Medicell International Ltd.) was prepared by boiling for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate, 1mM EDTA, followed by rinsing thoroughly with dH_2O. The tubing was stored at 4°C in 50% (v/v) ethanol and was rinsed with dH_2O before use. The tubing was sealed with plastic clips and dialysis carried out at 4°C with stirring and several changes of buffer.

2.2.6 Buffers

TE buffer: 10mM Tris-HCl (pH7.5), 1mM EDTA; autoclaved.

10 x TBE buffer: 0.9M Tris-borate (pH8.3), 20mM EDTA; unsterilised.

10 x TAE: 0.4M Tris-acetate (pH7.5), 20mM EDTA; unsterilised.

20 x SSC: 3M NaCl, 0.3M Na$_3$citrate, adjusted to pH7.0 with NaOH; unsterilized.

10 x PBS: 15mM KH_2PO_4, 1.5M NaCl, pH7.2; autoclaved.

10 x TBS: 0.5M Tris-HCl, 1.5M NaCl, pH7.5; autoclaved.

2.3 Microbiological Methods

All procedures were performed under conditions of "Good Microbiological Practice" as recommended by the U.K. Genetic Manipulation Advisory Group.

2.3.1 Growth and storage of E.coli

E.coli cells were grown at 37°C, on inverted agar plates or with continuous shaking for liquid cultures. The growth of liquid cultures was monitored by measurement of $O.D._{600nm}$. Strains of E.coli which did not carry plasmids were grown on LB-agar plates or in liquid LB. Strains carrying plasmids which conferred ampicillin resistance were grown on LB/ampicillin agar plates or in liquid LB/ampicillin. Agar plates and starter cultures were stored at 4°C and were viable for up to one month. E.coli cells were stored in the long term in 15% (v/v) glycerol, 85% (v/v) LB at -70°C.

2.3.2 Growth and storage of yeast

Yeast cells were propagated as described in Sherman et al (1983). Wild type strains were grown at 30°C, and rna2-1 and rna2-4 strains at 23°C, on inverted agar plates or with continuous shaking in liquid culture. The growth of liquid cultures was monitored by measurement of OD_{600nm}.

Strains not carrying plasmids were grown on YPDA-agar plates and in YPDA liquid medium. Strains transformed with plasmids were grown on YMM agar and in YMM liquid culture, supplemented with the appropriate amino acids and uracil, in order to select for the presence of the plasmid. Permanent yeast stocks were stored at -70°C in 15% (v/v) glycerol, 85% (v/v) YPDA.

2.4 Nucleic Acid Methods

2.4.1 Storage of nucleic acid

Plasmid DNA was stored at 4°C in dH_2O or TE . DNA oligonucleotides and RNA (tRNA, total yeast RNA, *in vitro* synthesised RNA) were stored at -20°C.

2.4.2 Quantitation of nucleic acids

Accurate estimation of concentrations of nucleic acids in solution were obtained by measuring OD_{260nm} using a Perkin-Elmer 320 spectrophotometer. Rough estimates of nucleic acid concentration were obtained by electrophoresing samples on an ethidium bromide-containing agarose gel (section 2.4.6) and comparing the fluorescence of the sample, when illuminated by UV light, with a concentration standard.

2.4.3 Precipitation of nucleic acids with ethanol

Nucleic acids were precipitated from aqueous solutions by addition of sodium acetate, pH5.2, to a final concentration of 300mM, followed by 2-5μg of carrier tRNA where necessary, and 2.5 volumes of ice cold ethanol. Precipitates were allowed to form for at least ten minutes on ice (in 1.5ml tubes) or for at least one hour at -20°C (for larger volumes). The precipitated nucleic acid was collected by centrifugation at 13,800 g at 4°C for fifteen minutes in a microcentrifuge (volumes less than 1.5ml) or at 16,000g at 4°C for thirty minutes in a Sorvall HB-4 rotor. Pellets were washed by resuspension in 70% ethanol and were recovered by centrifugation as above. The pellet was dried by allowing ethanol to drain from the

inverted tube, followed by brief incubation in a 65°C incubator, or under vacuum.

2.4.4 Deproteinisation of nucleic acid solutions

Aqueous nucleic acid solutions were deproteinized by addition of an equal volume of a 25:24:1 mixture of phenol, chloroform and isoamyl alcohol followed by vortexing. The aqueous and organic phases were separated by centrifugation, (in a microcentrifuge or at 3,000rpm in a bench top centrifuge with swing-out rotor), for one to ten minutes at room temperature. The aqueous phase was recovered and re-extracted until no further material was visible at the interface after centrifugation. If necessary, residual phenol and chloroform in the aqueous phase were removed by ethanol precipitation of the nucleic acid. Phenol was redistilled and stored under dH_2O at -20°C. Phenol used for DNA extractions was equilibrated with 0.5M Tris pH7.5 and then mixed with chloroform and isoamyl alcohol and stored under TE (section 2.2.6) at 4°C. Phenol-chloroform-isoamyl alcohol for RNA extractions was equilibrated with dH_2O and stored at 4°C.

2.4.5 Restriction endonuclease digestion of DNA

DNA was incubated with a greater than 2-fold excess of restriction endonuclease in the buffer recommended by the manufacturer of the enzyme. AflIII was incubated with DNA in a buffer containing 50mM NaCl, 10mM $MgCl_2$, 10mM β-mercapto ethanol, 10mM Tris-HCl pH7.5. Incubations were at 37°C for at least one hour. The progress of digestion was examined by agarose gel electrophoresis (section 2.4.6) and reactions stopped by freezing or phenol-chloroform extraction.

2.4.6 Agarose gel electrophoresis

Horizontal slab gels contained 0.8-1.5% agarose in TAE (section 2.2.6) buffer. The agarose was dissolved by boiling, and the solution was allowed to cool to 50°C before addition of ethidium bromide to 0.5μg/ml, immediately prior to pouring. Preparative gels and most analytical gels

were 20x20x0.7cm, while 13x9x0.4cm gels, with paper wicks were used for rapid screening purposes. Before loading samples, 0.2 sample volumes of loading buffer (50% glycerol, 0.25% bromophenol blue) was added to each sample. Gels were elecrophoresed at 5-15 V/cm in TAE. Preparative gels were shielded from light in order to prevent light-induced nicking of DNA in the presence of ethidium bromide, or were run without ethidium bromide, and stained subsequently in the dark. Nucleic acids were visualised by illumination from below with short wavelength (254nm) UV light. Gels were photographed though a red filter using Ilford HP5 5x4 inch negative or Polaroid 667 positive film.

2.4.7 Recovery of DNA fragments from agarose gels

2.4.7.1 Ground glass procedure

DNA was recovered from agarose gels by addition of sodium iodide to excised gel pieces, to dissolve the agarose, followed by addition of ground glass to precipitate DNA, using the 'GENECLEAN' kit (B10 101 Inc), according to the manufacturer's instructions.

2.4.7.2 Electroelution into a trough

This procedure is based on a method described in Maniatis *et al* (1982). Following electrophoresis DNA bands were visualised by illumination with long wavelength UV light in order to minimise damage to the DNA in the presence of ethidium bromide. A trough was cut in the gel directly ahead of the desired band (in the direction of electrophoresis), and dialysis tubing inserted into the trough to form the floor and far wall to the trough. The gel was then replaced in the electrophoresis tank, and the electrophoresis buffer removed until the surface of the buffer was level with, but not above, the top of the gel. The trough was filled with TAE (section 2.2.6) and electrophoresis resumed for two minutes. The polarity was briefly reversed to release DNA from the dialysis tubing and the buffer recovered from the trough. The gel was then illuminated with UV light to

check that the band of interest had migrated into the trough. DNA was recovered from the buffer in the trough by extraction with phenol-chloroform and precipitation with ethanol.

2.4.7.3 Electroelution into a dialysis bag

Following electrophoresis the DNA band of interest was visualised under UV light and excised from the gel with a scalpel. It was then inserted into a dialysis bag and the minimum volume of TAE necessary to cover the gel slice was added. The bag was placed in a small horizontal gel electrophoresis tank and submerged in TAE. The current was then switched on, and electroelution of the DNA from the gel slice allowed to continue for thirty minutes. The current polarity was then briefly reversed and the liquid in the bag recovered. Small pieces of agarose were removed by microcentrifugation for one minute, and DNA recovered by extraction with phenol-chloroform and precipitation with ethanol.

2.4.8 Denaturing acrylamide gel electrophoresis

Denaturing acrylamide gels contained 6-20% acrylamide (19:1 acrylamide: bis acrylamide), 8 M urea and 1xTBE (section 2.2.6), final concentrations. Gel stocks containing acrylamide and urea were made up at 1.1x final concentration and were deionized and stored at 4°C. Gels were prepared by mixing 45ml of the stock solution with 5ml of 10xTBE. 10% (w/v) ammonium persulphate (freshly prepared; 0.3ml) and TEMED (30µl) were added as polymerization catalysts. The gels were poured between two clean glass plates (42 x 23 cm) which were separated along the two long edges with 1cm wide strips of 0.4mm thick Plastikard (Slater's Plastikard) and sealed with PVC tape. Wells of width 0.5-1 cm were formed by inserting a Plastikard comb into the top of the gel after pouring. Polymerisation was allowed to proceed at room temperature for at least one hour, following which the gel was run, or stored overnight at 4°C.

To run the gel, the PVC tape sealing the bottom of the gel was removed, the comb withdrawn and the gel placed in a vertical gel electrophoresis tank which contained 1xTBE. To remove any small pieces of acrylamide the

wells were flushed out with a syringe. The gel was pre-electrophoresed for 30-60 minutes at 35W. Prior to loading samples two volumes of gel loading buffer (10M urea, 1xTBE, 0.1% (w/v), 0.1% (w/v) xylene cyanol) were added to each sample, which was then incubated at 65°C for three minutes. Samples were loaded using a drawn out glass capillary. Electrophoresis was at 35W and was continued until the dyes had migrated the desired distance.

2.4.9 Elution of ^{32}P-labelled RNA from denaturing polyacrylamide gels

^{32}P-labelled RNA was electrophoresed on 42 x 23cm or on 16 x 16cm denaturing polyacrylamide gels. A brief exposure of the gel, which was labelled with radioactive ink of the appropriate specific activity was made, and the band of interest excised from the X-ray film. The X-ray film was aligned with the gel and the band beneath the hole in the film excised, cut into several pieces and placed in a 1.5ml polypropylene tube. Elution buffer (0.5M ammonium acetate, 1mM EDTA, 0.5% SDS; 0.3ml) was added to the gel pieces and the tube incubated on a rotating wheel for thirty minutes at 37°C. The gel pieces were removed by centrifugation for fifteen seconds in a microcentrifuge and the supernate removed and re-centrifuged for two minutes to remove any small pieces of polyacrylamide. RNA was recovered from the eluate by extraction with phenol-chloroform and precipitation with ethanol, without added salt.

2.4.10 Ligation of DNA fragments

Ligations of linearized vectors to DNA fragments with cohesive ends were performed with a vector: insert molar ratio of 1:3 and with a final vector concentration of 20μg/ml. The DNAs, in dH_2O were added to a 20μl final reaction volume, containing 50mM TrisHCl pH7.5, 10mM $MgCl_2$, 10mM DTT, 50mg/ml BSA (fraction V), 1mM ATP and two units T4 DNA ligase. Reactions were incubated overnight at 14°C.

2.4.11 Filling in recessed 3' ends of DNA

Recessed 3' ends of DNA, generated by restriction endonuclease cleavage were filled in using the large fragment of E.coli polymerase 1 (the Klenow fragment). The DNA fragment (0.1-2μg) was incubated for one hour at 37°C with two units of this enzyme and 0.1mM each of dATP, dCTP, dGTP and dTTP, in a buffer which contained 50mM Tris-HCl pH7.5, 10mM $MgCl_2$ 6mM DTT. The reaction was stopped by extraction with phenol-chloroform, or heating to 65°C for fifteen minutes.

2.4.12 Transformation of E.coli

2.4.12.1 Preparation of competent cells

The method of preparation of E.coli cells which are competent for transformation with DNA is a modification of the method of Hanahan (1983). A single colony was picked and grown overnight in 10ml of LB. The starter culture (5ml) was inoculated into 100ml of LB and incubated with shaking at 37°C until an OD_{600nm} of 0.5 was reached. The culture was chilled on ice water and then transferred to pre-chilled 50ml centrifuge tubes. Cells were sedimented by centrifugation at 1000xg for five minutes at 4°C and were resuspended in 50ml of cold TFB1 (100mM RbCl, 50mM $MnCl_2$, 35mM sodium acetate pH5.8, 10mM $CaCl_2$, 15% (v/v) glycerol; filter sterilized) and incubated on ice for thirty minutes. The cells were re-centrifuged, as above, resuspended in 4ml of cold TFB2 (10mM MOPS pH6.8, 10mM RbCl, 75mM $CaCl_2$, 15% (v/v) glycerol; filter sterilized) and incubated on ice for twenty minutes. They were then used directly for transformation, or snap-frozen in liquid nitrogen and stored at -70°C in 0.4ml aliquots.

2.4.12.2 Transformation of competent E.coli

Competent cells (200μl) were mixed with aliquots of ligation reactions or 100ng of plasmid DNA and incubated on ice for thirty minutes. The suspension was transferred to a small glass test tube and shaken in a 42°C water bath for two minutes. It was then diluted with 0.8ml LB and incubated at 37°C on a rotating wheel for at least one hour. Cells were

plated out on LB-agar/ampicillin plates and incubated overnight at 37°C, to allow colony formation.

2.4.13 Transformation of yeast

2.4.13.1 Transformation of yeast spheroplasts

This method is based on that used by Beggs (1978). A 50ml culture of cells was grown in YPDA, overnight, to late log phase and cells harvested by centrifugation (3,000 rpm in the bench top centrifuge for five minutes). Cells were washed with dH_2O by resuspension and centrifugation, as above, and were resuspended in 20ml of 1M sorbitol, 25mM EDTA pH8.0, 50mM DTT. This suspension was incubated with gentle shaking at the growth temperature of the culture for fifteen minutes and cells harvested by centrifugation as above. The cell pellet was washed with 1.2M sorbitol by resuspension and centrifugation and resuspended in 20ml of 1.2M sorbitol, 10mM EDTA, 100mM sodium citrate pH5.8, to which β-glucuronidase (Sigma; filter sterilized) was added to 1% (v/v). The suspension was incubated at the growth temperature of the culture with very gentle shaking for sixty to one hundred minutes. Spheroplast formation was monitored by diluting aliquots of cells in dH_2O and observing lysis under the light microscope. Spheroplasts were harvested by centrifugation at 2,000rpm in the bench top centrifuge and were washed three times with 1.2M sorbitol by resuspension and centrifugation at 2,000rpm.

Spheroplasts were resuspended in 0.5ml of 1.2M sorbitol, 10mM $CaCl_2$ and 50µl aliquots incubated with 1-10µg of DNA (up to 15µl), at room temperature for fifteen minutes. The mixture was diluted with 0.5ml of 20% PEG 4000, 10mM $CaCl_2$, 10mM Tris-HCl pH7.5 and incubated for five minutes, followed by brief centrifugation in the microcentrifuge. The spheroplast pellet was resuspended in 100µl of YPDAS and incubated at the growth temperature of the yeast strain for several hours. Dilutions of the suspension were made in 1.2M sorbitol. The dilutions were added to 7ml YMMS top agar (cooled to 45°C immediately before use), containing the appropriate amino acids and uracil and immediately spread onto the surface of YMMS agar plates which contained the same amino acids. The top agar

was allowed to solidify and the plates incubated at the growth temperature of the strain, until transformant colonies were visible (usually four to five days).

2.4.13.2 Transformation of yeast cells

This procedure is the same as that of Ito et al (1983). A culture was grown overnight to stationary phase in YPDA at 30°C and cells harvested by centrifugation at 3,000 rpm in the bench top centrifuge for five minutes. The cell pellet was washed by suspension in TE and centrifugation as above, resuspended in TE, and LiCl (2.5M stock) added to give a final concentration of 0.1M. The cells were incubated at 30°C for one hour with gentle shaking and 5μg of plasmid DNA was then added to 200μl of the cell suspension. The mixture was incubated without shaking for thirty minutes at 30°C and 0.7ml of 50% (w/v) PEG4000 added. This suspension was incubated for one hour at 30°C and then heated at 42°C for five minutes. The cells were harvested by centrifugation for twenty seconds in the microcentrifuge, washed with dH_2O by resuspension and centrifugation. They were then either resuspended in 0.1ml dH_2O and plated directly into YMM-agar plates, supplemented with the appropriate amino acids and uracil to select transformants or were resuspended in 1ml YPDA and grown for several hours at 30°C prior to plating in dH_2O as above. The plates were incubated at 30°C and transformant colonies were usually visible after three to four days.

2.4.14 Preparation of plasmid DNA from E.coli cells

2.4.14.1 Small scale, alkaline extraction of plasmid DNA

This procedure is based on that of Birnboim and Doly (1980). Cultures (3ml) of E.coli transformants were grown overnight on LB/ampicillin, and 1.5ml of the culture centrifuged in the microcentrifuge for thirty seconds to sediment the cells. The pellet of cells was resuspended in 100μl of lysis solution (10% w/v glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA, 4mg/ml lysozyme; freshly prepared) and incubated on ice for thirty minutes. 200μl of 1% (w/v) SDS, 0.2M NaOH (freshly prepared) was then added and

incubation on ice continued for five minutes, followed by addition of 150µl of 3M sodium acetate, pH5.2, and further incubation on ice for at least sixty minutes. Cell debris and chromosomal DNA were removed by spinning in the microcentrifuge for ten minutes and 400µl of supernate was carefully removed. This supernate was extracted with phenol-chloroform and plasmid DNA precipitated with ethanol, without addition of further sodium acetate. The pellet from the ethanol precipitation was resuspended in 50µl of dH_2O and RNase A (10mg/ml stock solution prepared by boiling for fifteen minutes to inactivate DNases; stored at -20°C) added to 100µg/ml final concentration. The digestion of RNA was allowed to proceed for thirty minutes at 37°C, prior to digestion of the plasmid DNA with restriction endonucleases.

2.4.14.2 Large scale CsCl preparation of plasmid DNA

This method is a modified form of that used by Guerry et al (1973). a 500ml culture of E.coli transformants picked from a single colony was grown overnight to stationary phase in LB/ampicillin. The cells were harvested at 5000rpm for ten minutes (Sorvall GS-3 rotor) at 4°C and resuspended in 6ml of 25% (w/v) sucrose, 50mM Tris-HCl pH8.0, 62.5mM EDTA and transferred to 30ml polycarbonate centrifuge tubes. Lysozyme solution (1ml, 10mg/ml lysozyme, in the same buffer) was added and the tubes incubated at room temperature for five minutes, after which 1ml of 0.5M EDTA, pH8.0, was added and incubation continued for a further ten minutes at room temperature. The tubes were then transferred to ice, and 14ml of cold 'Triton mix' (0.1% (v/v) Triton X-100, 50mM Tris-HCl pH8.0, 10mM EDTA, 10% (w/v) glucose) was added, and the suspension gently mixed. After ten minutes on ice, or when the cell lysate had become viscous due to release of chromosomal DNA from cells, the lysate was centrifuged at 17,000 rpm for forty minutes at 4°C in an SS34 rotor. The supernate was removed and 0.95g CsCl and 50µl 10mg/ml ethidium bromide added for each ml of supernate. The supernate was then transferred to two 12ml polyallomer tubes which were sealed. The tubes were centrifuged in a Ti50 rotor at 37,000 rpm and 18°C for at least forty hours. DNA was visualised under long-wavelength UV light and recovered by piercing the tube with a 19-guage needle and syringe. Ethidium bromide was removed by six extractions with

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butan-1-ol (equilibrated with CsCl-saturated TE buffer). CsCl was removed by dialysis for several hours against TEN buffer (TE plus 100mM NaCl), followed by overnight dialysis against TE buffer. The DNA was deproteinized by extraction with phenol-chloroform, precipitated with ethanol and redissolved in TE or dH_2O.

2.4.15 Measurement of radioactivity in nucleic acids

This method is a rapid and simplified version of that in Maniatis et al (1982). ^{32}P-labelled nucleic acids were spotted onto GF/C filters and the number of counts on the filters determined by Cerenkov counting, or estimated with a hand-held Geiger counter. The filters were then placed onto a vacuum filtration apparatus and 50ml of 10% TCA, followed by 50ml of 5% TCA, 50mM sodium pyrosphate passed through the filter. The amount of radioactivity on the filter was then determined as above. The ratio of the two different figures, before and after TCA precipitation, gave the percentage incorporation of radioactivity into nucleic acids. Oligonucleotides of less than twenty nucleotides in length are not efficiently retained on GF/C filters using this procedure.

2.4.16 Radio-labelling of DNA probes by random priming

This method is based on that of Feinberg and Vogelstein (1983). The random priming reaction mix was prepared by adding reagents in the following order: dH_2O (to give a final volume of 50µl), 10µl oligo-labelling buffer (see below), 1µl of 20mg/ml BSA, up to 32.5µl DNA solution (denatured by boiling for four minutes followed by rapid cooling on ice-water; 50-100ng DNA), 20µCi α-^{32}P-dCTP, two units of Klenow enzyme. The reaction mix was incubated at room temperature for 4-6 hours and the reaction terminated by boiling prior to hybridization (see section 2.4.20). Incorporation of ^{32}P-CTP into DNA was assayed by TCA precipitation.

Oligo-labelling buffer was prepared as follows. Solutions A, B and C were mixed in the ratio 1:2.5:1.5

Solution O: 1.25M Tris-HCl pH8.0, 125mM $MgCl_2$; stored at 4°C

Solution A: 1ml solution O + 18µl β-mercaptoethanol + 5µl each dATP, dCTP, dGTP (each 0.1M in 3mM Tris-HCl pH 7.0, 0.2mM EDTA); stored at -20°C).

Solution B: 2M HEPES-NaOH pH6.6; stored at 4°C

Solution C: Random sequence hexadeoxynucleotides in TE buffer, 90 OD_{260nm} units/ml; stored at -20°C.

2.4.17 5' end-labelling of DNA oligonucleotide probes

This procedure is modified from the procedure of Maxam and Gilbert (1980). The labelling reaction contained 20 pmol of oligonucleotide, 20µCi of γ-^{32}P-ATP, two units of T4 nucleotide kinase and 1µl of 10 x kinase buffer (700mM Tris-HCl pH7.5, 100mM $MgCl_2$, 1mM KCl, 50mM DTT) in a final volume of 10µl. The reaction was incubated at 37°C for thirty minutes. It was then added directly to hybridization solution or, when necessary, unincorporated γ-^{32}P-ATP removed by gel filtration, using a Sephadex G-25 column (Pharmacia, NAP10), according to the manufacturer's instructions.

2.4.18 Southern blotting from agarose gels

DNA was transferred from agarose gels to nitrocellulose filters according to the method of Southern (1975). Following electrophoresis and photography of the gel, the gel was soaked in 500ml of Southern denaturation buffer (1.5m NaCl, 0.5M NaOH) for one hour at room temperature, with gentle shaking. The gel was then neutralised by a further one hour incubation, with gentle shaking, in Southern neutralization buffer (1.5M Tris-HCl pH8.0, 1.5M NaCl). A double sheet of Whatman 3MM paper was wetted in a reservoir containing 10 x SSC buffer and draped over a glass plate with both ends dipping into the reservoir. The gel was then placed onto the 3MM paper on the glass plate with its underside facing upwards, and any regions of the gel from which DNA was not to be transferred were covered with Saran wrap. The exposed portion of the gel was then covered with a piece of nitrocellulose membrane (BA85, Schleicher and Schuell) which had been pre-wetted in 2xSSC, taking care to remove any air bubbles

between the gel and filter. Two pieces of Whatman 3MM paper, pre-wetted in 2xSSC were placed on top of the nitrocellulose membrane, and on top of these a 5-10cm high stack of paper towels. A glass plate, weighted down with a 500g weight was placed on top of the paper towels, and transfer was allowed to proceed overnight, after which the nitrocellulose was removed, rinsed in 6xSSC for two minutes and baked at 85°C under vacuum.

2.4.19 Transfer of DNA from E.coli colonies to nitrocellulose filters

This procedure, for screening E.coli colonies to identify those carrying recombinant plasmids derives from Grunstein and Hogness (1975). Duplicate plates, carrying colonies streaked in a grid pattern were grown overnight. Routinely, 200 colonies were screened at one time. A circular nitrocellulose filter (BA85, Schleicher and Schuell), with orientation marks on one side, was placed gently on one plate of each pair, while the other was saved, as a master plate. The filter was removed and placed, colony side upwards upon a piece of filter paper, pre-soaked in Southern denaturation buffer (section 2.4.18) for 5 minutes, and then transferred to a similar filter, pre-soaked in Southern neutralization buffer (section 2.4.18) for a further 5-10 minutes. The filter was washed briefly in 2xSSC, dried on filter paper at 37°C for ten minutes and then baked for two hours at 80°C under vacuum.

2.4.20 Hybridisation of Southern and colony blots

Filters carrying DNA transferred from an agarose gel or from E.coli colonies were sealed into plastic bags or placed in plastic airtight boxes with 10-30ml of prehybridization buffer (2xSSC, 10xDenhardts solution [0.2% Ficoll, 0.2% (w/v)BSA, 0.2%(w/v) polyvinylpyrollidone], 2% SDS, 0.1mg/ml single-stranded salmon sperm DNA; pre-heated to 65°C) and incubated at 65°C for greater than two hours. The probe (section 2.4.16) was denatured by boiling for four minutes, followed by rapid cooling on ice-water and was added to the liquid in the bag or box. Hybridization was allowed to continue overnight at 65°C, and the filter then removed from the hybridization solution and washed three times for five minutes with 500ml of 2xSSC at room temperature. If necesary two further washes at 65°C in

2xSSC, 0.5% SDS were performed and the filter air-dried on filter paper and exposed to X-ray film.

2.4.21 Northern blotting from denaturing polyacrylamide gels

Following electrophoresis (section 2.4.9) the gel plates were separated leaving the gel on one plate, and regions of the gel which did not contain samples to be transferred were removed. The gel was lifted off the glass plate using dry filter paper, which was then wetted by placing on filter paper which had been soaked in 0.5xTBE buffer. Three further pieces of filter paper, of the same dimensions, two Scotchbrite pads and a piece of nylon membrane (Hybond-N, Amersham) cut to dimensions slightly larger than those of the gel, were all soaked in 0.5xTBE. A 'sandwich' was then constructed which consisted of one Scotchbrite pad on top of which was placed a piece of filter paper, followed by the gel on its piece of filter paper, the nylon membrane, two further pieces of filter paper and the second Scotchbrite pad. Care was taken to ensure that there were no air bubbles between any of the layers of the 'sandwich', which was then inserted into the plastic cassette of a Trans-Blot apparatus (Bio-Rad). The cassette was placed in the electrophoresis tank of the Trans-Blot apparatus with the gel side oriented towards the anode, and transfer performed in 0.5xTBE for one hour at 200mA. The buffer in the tank was cooled by metal or plastic coils through which tap water flowed. Following transfer, the nylon filter was removed and dried for ten minutes at 37°C on filter paper with the RNA side upwards. The filter was then covered with Saran Wrap and placed RNA side downwards on a UV transilluminator for five minutes, in order to covalently link the nucleic acid to the filter.

2.4.22 Hybridization of Northern blots with oligonucleotide probes

This method is adapted from that of Church and Gilbert (1984). The nylon membrane (prepared as in section 2.4.21) was wetted in dH_2O, and placed in a plastic airtight box containing 20-30ml of hybridization solution (0.5M sodium phosphate, pH7.0, 7% SDS, 1mM EDTA). The membrane was incubated in this solution at 28-37°C for at least one hour and was

then transferred to a heat sealable plastic bag. The oligonucleotide probe (section 2.4.17) in 1.5ml of 10mM sodium phosphate buffer was added directly to 10-20ml of hybridization buffer (pre-warmed to 28-37°C) and the hybridization buffer containing the probe was added to the plastic bag containing the filter. The bag was sealed and placed in a water bath at 28-37°C (depending on the melting temperature of the DNA-RNA hybrid) overnight, with continuous shaking. The membrane was then removed from the bag and washed twice at 28-37°C for ten minutes in 0.5M sodium phosphate pH7.0, 5% SDS, 1mM EDTA. The membrane was exposed, without drying, between two sheets of Saran Wrap.

2.4.23 In vitro transcription reactions

In vitro transcription of DNA using SP6 polymerase (Melton et al, 1984) or T7 polymerase (Davanloo et al, 1984) was performed on DNA which was linearised with restriction enzymes. The reaction mixture contained 0.1-0.2μg DNA, 0.5mM ATP, CTP and GTP, 25μM UTP, 20-40μCi α-^{32}P-UTP, 10 units RNasin (Promega Biotech), 5 units of the appropriate RNA polymerase and 1ml of 10xtranscription buffer (400mM Tris-HCl pH7.5, 60mM $MgCl_2$, 100mM DTT [plus 100mM NaCl for T7 polymerase]). The reactions were performed in a final reaction volume of 5 or 10μl for 15-30 minutes and were stopped on ice. Incorporation of radioactive label into RNA was measured by TCA precipitation (see section 2.4.15) and the transcripts stored at -20°C.

2.4.24 RNase H digestions

SP6 or T7 transcribed RNA (section 2.4.23) was hybridized to 0.2 nmol of oligonucleotide in the manufacturers' recommended RNase H digestion buffer by incubation at 65°C for three minutes, followed by incubation at 25°C for thirty minutes, in a volume of 10μl. One unit of E. coli RNase H was added and incubation continued at 37°C for 30-60 minutes. The reaction was stopped by placing on ice.

2.5 In vitro Splicing

2.5.1 Preparation of yeast splicing extract

This procedure is slightly modified from that of Lin et al (1985). A 1L or 1.5L culture of yeast cells was grown at 30°C (wild type strains) or 23°C (temperature sensitive strains) to an OD_{600nm} of 0.4 to 0.6 and harvested by centrifugation (5,000rpm for ten minutes at room temperature in a Sorvall GS-3 rotor). The cells were washed in 50ml of 50mM potassium phosphate pH7.5, by resuspension and centrifugation (in the bench top centrifuge, 3,000rpm for five minutes) and then resuspended in 50ml of lyticase buffer (50mM potassium phosphate, 1.2M sorbitol, 30mM DTT), to which 1 to 1.5ml of lyticase (Sigma) was added. Digestion of cell walls was allowed to proceed for up to 45 minutes at the growth temperature of the strain, with very gentle shaking. Spheroplast formation was monitored by observing lysis upon dilution of small aliquots of cells into dH_2O, and when greater than 80% of cells were clearly sensitive to lysis, the spheroplasts were sedimented (2,000rpm, five minutes in the bench top centrifuge at room temperature). They were then washed twice with 50mM potassium phosphate pH7.5, 1.2M Sorbitol by resuspension and centrifugation (2,000rpm, five minutes in the bench top centrifuge at room temperature), resuspended in 200ml of YPDAS, and incubated with very gentle shaking at the appropriate growth temperature for 1.5 to 2 hours. The spheroplasts were harvested by centrifugation (3,000rpm for ten minutes at room temperature in a Sorvall GSA rotor), washed in 50mM sodium phosphate pH7.5, 1.2M sorbitol by resuspension and centrifugation and resuspended in cold buffer A (10mM HEPES-KOH pH7.0, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT, 1mM PMSF; 1ml per gram of spheroplasts). The suspension was transferred to a cold Dounce homogenizer and incubated on ice for five minutes to allow osmotic swelling. Subsequent steps were performed in the cold room. The spheroplasts were lysed with twelve strokes of a tight fitting Teflon pestle, on ice, and transferred to a small glass beaker. Cold KCl was added slowly, to a final concentration of 200mM and the mixture stirred on ice for thirty minutes. The lysate was then cleared by centrifugation (17,000rpm for thirty minutes at 4°C in a Sorvall SS34 rotor) and the supernate subjected to a further centrifugation (37,000 rpm for 1 hour at

4°C in a Ti50 rotor). The supernate from the second centrifugation was then either dialysed against two litres of D buffer (50mM KCl, 20mM HEPES-KOH pH7.0, 0.2mM EDTA, 0.5mM DTT, 20% (v/v) glycerol) or treated with ammonium sulphate (section 2.5.2). Following dialysis the protein extract was removed from the dialysis bag and centrifuged for ten minutes at 4°C in the microcentrifuge. The supernate was divided into 50µl aliquots, snap frozen in liquid nitrogen and stored at -70°C.

2.5.2 Ammonium sulphate treatment of splicing extracts

In some cases the supernate of the 37,000rpm spin was treated with ammonium sulphate according to the procedure of Vijayraghavan et al (1986). Ammonium sulphate powder (enzyme grade, ground with a mortar and pestle; 0.246g/ml of supernate) was added slowly to the supernate, with continuous stirring, on ice. The stirring was allowed to continue for a further thirty minutes on ice, and the extract was then centrifuged at 15,000rpm for twenty minutes at 4°C in a Sorvall SS34 rotor. The protein precipitate was redissolved in one tenth of the original extract volume of splicing extract dialysis buffer (section 2.5.1), on ice, and dialysed, centrifuged and frozen in the same way as untreated splicing extract. This fraction of splicing extract is referred to as 35P1.

2.5.3 In vitro splicing

In vitro splicing was performed according to the procedure of Lin et al (1985), with slight modifications. Reactions were set up as follows: 5µl of splicing extract, or 2.5µl each of splicing extract and 35P1, were allowed to warm to room temperature. A mix (3µl) containing 10% PEG, 25.6% IgG buffer (250mM potassium phosphate, 67mM sodium citrate, 17% (v/v) glycerol, pH7.0), 200mM potassium phosphate, pH7.5, 8.3mM $MgCl_2$, was then added. In the case of prp2 extracts to be heat inactivated, the mixture was then incubated for up to one hour at 32°C. 1µl of dH_2O (or PRP2 protein preparation to be assayed for PRP2 activity, section 4.4), followed by 1µl of a 1:5 to 1:20 dilution of SP6 or T7 RNA transcript, in 2mM ATP,

was then added, and the reaction incubated at 25°C for up to one hour. The reaction was stopped by dilution with 175µl of 50mM sodium acetate pH5.2, 1mM EDTA, 0.1% SDS, 20µg/ml tRNA, followed by extraction with phenol/chloroform and precipitation of RNA with ethanol. The precipitated RNA was analysed by denaturing polyacrylamide gel electrophoresis (section 2.4.9).

2.5.4 Non-denaturing gel electrophoresis

Non-denaturing gel electrophoresis was performed by a slight modification of the method of Pikielny et al (1986). The gel mixture for non-denaturing gels contained 3% (w/v) acrylamide (60:1 acrylamide bisacrylamide), 0.5% (w/v) agarose, 1xTBE. This was prepared by dissolving the agarose in TBE by boiling, and allowing the gel to cool to approximately 65°C. The gel was then degassed, acrylamide/bisacrylamide stock solution and polymerising agents (see below) were addedand the gel was immediately poured between 16cmx16cm glass plates separated by 1.5mm thick spacers. The polymerising agents used were APS and 3-dimethyl-amino-propionitril (Fluka Chemie A.G.; 0.05% and 0.4% final concentration, respectively). The gel was allowed to polymerize for one hour at room temperature, and was then transferred to the cold room and pre-electrophoresed for one hour at 150V.

Samples were placed on ice and an equal volume of cold Q buffer (400mM KCl, 2mM magnesium acetate, 20mM EDTA, 64mM Tris-HCl pH 7.5), plus 6µg of yeast total RNA per 10µl sample, was added. The mixture was incubated on ice for ten minutes, 0.25 volumes of 5xloading buffer (50% (v/v) glycerol, 2.5xTBE, 0.05% (w/v) each xylene cyanol and bromophenol blue) added, and the sample loaded on the gel. Electrophoresis was for four to sixteen hours at 50-200V, with recirculation of buffer.

2.5.5 Elution of spliceosomal complexes from non-denaturing gels

Following electrophoresis, gels were autoradiographed at 4°C with pre-flashed film, with markers of radioactive ink. The X-ray film was then aligned with the gel and the band of interest excised, cut into several

small pieces and placed in a dialysis bag with 0.5-0.7ml TBE. The bag was placed in a horizontal gel electrophoresis tank containing pre-cooled (to 4°C) TBE, and electroelution performed for two hours at 150V and 4°C. The current polarity was reversed for one minute, and the liquid withdrawn from the dialysis bag.

2.6 Protein Methods

2.6.1 Measurement of protein concentration

To estimate protein concentration, 2µl of dilutions of the sample in dH_2O were spotted onto Whatman no. 1 paper, alongside BSA concentration standards. The filter was dried at 65°C for five minutes, and briefly immersed in 25% (v/v) propan-2-ol, 10% (v/v) acetic acid, 0.1% Coomassie Brilliant Blue R250 (Bio Rad). The filter was washed with tap water until background staining of the filter was low, and the protein concentration estimated by comparing the intensity of staining of the protein spots with the standards.

2.6.2 SDS-polyacrylamide gel electrophoresis

Proteins were separated by SDS-discontinuous polyacrylamide gel electrophoresis, as described by Laemmli (1970). An 8.5% or 15% (w/v) acrylamide separating gel mix (see below, Table 2.4) was poured between two 16x16cm glass plates, which were separated by 1.5mm thick spacers and sealed with rubber tubing, until the liquid level was approximately 5 cm below the top of the notched plate. dH_2O was carefully layered onto the surface of the separating gel in order to form a straight interface, and the gel allowed to polymerise for thirty minutes. The dH_2O was then removed and stacking gel mix (Table 2.4) was poured between the plates upto the top of the notched plate, and a Teflon comb (with 12 0.5cm or 20 0.3cm wells) was inserted. The stacking gel was allowed to polymerise for at least thirty minutes, the comb and rubber tubing removed and the gel placed in a vertical gel electrophoresis tank containing SDS-PAGE running buffer (25mM Tris, 192 mM glycine pH8.3, 0.1% SDS). Samples were mixed with an equal

volume of SDS-gel loading buffer (125mM Tris-HCl pH6.8, 200mM DTT, 4% (w/v) SDS, 40% glycerol, 0.02% bromophenol blue; stored at 4°C), heated to 100°C for five minutes and loaded onto the gel. Electrophoresis was performed at 40-220V for 3-16 hours.

Table 2.4: Composition of SDS-polyacrylamide gel mixes

Stock Solutions	Separating Gels		Stacking Gel
	8.5%	15%	
30% (w/v) acrylamide	8.5ml	15ml	1.67ml
2.5% (w/v) bis-acrylamide	2.0ml	1ml	0.65ml
1M Tris-HCl pH 8.8	11.2ml	11.2ml	-
1M Tris-HCl pH 6.8	-	-	1.25ml
dH_2O	8.4ml	2.9ml	6.4ml
10% SDS	300µl	300µl	100µl
10% (w/v) APS	200µl	200µl	100µl
TEMED	20µl	20µl	10µl

2.6.3 Coomassie blue staining of protein gels

Following electrophoresis, the gel was transferred to a plastic container and incubated for thirty minutes at 37°C in Coomassie Blue staining solution (25% (v/v) propan-2-ol, 10% (v/v) acetic acid, 0.05% (w/v) Coomassie Brilliant Blue R250) with gentle agitation. The staining solution was removed, the gel rinsed very briefly in dH_2O to remove excess

staining solution and then immersed in destaining solution (25% (v/v) methanol, 20% (v/v) acetic acid) at room temperature, with gentle agitation. Small pieces of polyurethane foam were added to the destaining solution to adsorb Coomassie Blue, and destaining was continued until the background was low enough to clearly visualize the protein bands. The gel was dried at 80°C under vacuum.

2.6.4 Extraction of proteins from E.coli

Cells were harvested by centrifugation, (centrifugation conditions depending upon volume of culture) and were resuspended in dH_2O. They were lysed by addition of an equal volume of SDS-polyacrylamide gel loading buffer (section 2.6.2) and heating to 100°C for five minutes. The lysate was centrifuged for three minutes at room temperature to remove insoluble material and the supernate loaded onto an SDS-polyacrylamide gel with wells for analysis of the protein, or onto a preparative SDS-polyacrylamide gel (without wells) for purification of fusion proteins (see section 2.6.8.1).

2.6.5 Extraction of proteins from yeast

Yeast cultures were grown overnight in supplemented YMM until the OD_{600nm} was approximately 0.4 and cells were harvested by centrifugation (centrifugation conditions depending upon culture volume). The cells were washed in PBS (section 2.2.6), by resuspension and centrifugation (3,000rpm, for five minutes at room temperature in the bench top centrifuge) and resuspended in at least 0.4ml of lysis buffer (PBS, 0.1% NP40, 1mM PMSF). The cells were lysed by sonication on ice, at full power, for three periods of thirty seconds (with thirty seconds on ice between each period of sonication). The lysate was centrifuged in a microcentrifuge for thirty seconds at room temperature, and the supernate centrifuged for a further ten minutes at 4°C in a microcentrifuge. The supernate from this second spin was either used directly for immunoprecipitations, or analysed by SDS-PAGE (section 2.6.2).

2.6.6 Induction of fusion protein and PRP2 protein synthesis in E.coli with IPTG

Starter cultures (10ml) of E.coli carrying pUR fusion constructs, parent vector or pDR-PRP2 (section 4.2) were grown overnight at 37°C in LB/ampicillin. This starter culture was diluted 1:100 in fresh LB/ampicillin medium (up to 1L for preparative purposes), and incubated at 37°C until the OD_{600nm} had reached 0.4. IPTG was then added to give a final concentration of 0.5mM and the incubation continued for a further thirty minutes to one hour.

2.6.7 Induction of fusion protein synthesis in E.coli with indole-acetic acid

Induction of trpE fusion protein synthesis in E.coli cells (strain HB101) carrying pATH fusion constructs was performed by the method of Spindler et al (1984). Cells were grown overnight in M9+CA+trp/ampicillin medium at 37°C. This starter culture was then diluted 1:10 into M9+CA/ampicillin medium and incubated with vigorous shaking for one hour at 37°C. IAA (1mg/ml in EtOH) was added to a final concentration of 5µg/ml and incubation continued for a further two hours with vigorous shaking at 37°C.

2.6.8 Induction of over-expression PRP2 protein in yeast with galactose

Yeast cell extracts containing high levels of PRP2 protein were produced from KY117 cells carrying pBM-PRP2 (section 4.1.2), or DJY40 cells carrying pKV-PRP2 (section 4.1.2). These plasmids contain the whole of the PRP2 gene under the control of the yeast GAL1 promoter. In order to induce over-expression of PRP2 protein, 10ml starter cultures of cells carrying these constructs, or parent vectors, were grown to stationary phase on yeast minimal medium, (supplemented with the appropriate amino acids to select for the plasmids), with 2% glucose as carbon source. Cells were then pelleted by centrifugation (4,000rpm, for 5 minutes in the bench top centrifuge), washed once with yeast minimal medium, containing 2% raffinose plus 2% galactose, and resuspended in 10ml of this medium. The cell

suspension was diluted 1:100 in the same medium (supplemented with the appropriate amino acids), and grown overnight at 30°C, until the OD_{600nm} was 0.4, whereupon they were harvested.

2.6.9 Purification of fusion proteins from E.coli

2.6.9.1 Purification by SDS-PAGE

A 200ml culture of E.coli was induced for fusion protein synthesis (section 2.6.6 or 2.6.7), a lysate prepared (section 2.6.4) and electrophoresed on a 1.5 mm thick, 8.5% SDS-polyacrylamide gel without slots. Following electrophoresis, vertical strips were cut from the centre and each edge of the gel, and stained with Coomassie Blue . The stained strips were then realigned with the main portion of the gel, and a horizontal strip of gel containing the fusion protein excised. This strip was cut into several pieces and placed in a dialysis bag with 3-5ml of SDS-PAGE running buffer. The dialysis bag was placed in a horizontal gel electrophoresis tank and submerged in SDS-PAGE running buffer. The fusion protein was eluted for four hours at 150V, followed by brief reversal of the current polarity to remove the protein from the dialysis tubing. The liquid in the bag was withdrawn and the fusion protein concentrated by centrifugation at 3,000rpm (benchtop centrifuge with fixed angle rotor) in a Centricon 30 microconcentrator (Amicon Corporation). When the volume of the eluted fusion protein solution was less than 1.5ml, the solution was snap frozen and stored at -70°C.

2.6.9.2 Purification of proteins by urea fractionation

β-galactosidase fusion proteins were purified by the procedure of Hall et al (1984). A 100ml culture of E.coli cells, carrying pUR fusion constructs was grown to an OD_{600nm} of 0.4 and fusion protein expression induced with IPTG (section 2.6.6). The cells were then harvested by centrifugation (3,000rpm, five minutes at 4°C in fixed angle bench-top centrifuge) and resuspended in 2ml of 15% sucrose, 50mM Tris pH8.0, 50mM EDTA. The cell suspension was transferred to a 30ml polycarbonate tube and 200µl of 10mg/ml lysozyme (freshly prepared) was added. The mixture

was incubated for forty minutes on ice, with intermittent gentle agitation, and 0.1% Triton X-100 in TE buffer (2.8ml) added. After a further five minute incubation on ice the cell lysate was centrifuged at 17,000rpm for fifteen minutes at 4°C (Sorvall SS-34 rotor). The pellet was resuspended in 2ml of 2M urea by sonication (in ten second bursts, with ten seconds on ice between each burst, continued until the pellet had dispersed) and centrifuged, as above. The pellet from this centrifugation was resuspended in 2ml of 6M urea, by sonication, and centrifuged, as above. The resulting supernatant was dialysed against 2L of PBS, and concentrated using a Centricon microconcentrator (section 2.6.9.1).

2.6.10 Immunisation of rabbits

All experiments on live animals were performed in compliance with the Cruelty to Animals Act (1986) under licence number 61400. Rabbits were females of the half-lop variety and were obtained from Froxfield Rabbits.

50-200μg of fusion proteins were used for the first injection into rabbits, and 30-50μg for subsequence injections. 1-2 volumes of adjuvant (Freunds complete adjuvant for the first injection, Freunds incomplete adjuvant for subsequent injections) was added to the fusion protein preparation, and the mixture vortexed until a stable emulsion was formed. The emulsion was injected subcutaneously at several sites on the first injection, and at a mixture of intra-muscular and subcutaneous sites for subsequent injections. Blood was collected from the ear vein prior to the first injection and 12-14 days after the second and subsequent injections. 12-14 days after the final injection the rabbit was anaesthized with Sagatal (May and Baker Ltd.), and blood (100-150ml) withdrawn directly from the heart, using a 19-gauge needle and syringe. The rabbit was then killed with a further injection of Sagatal.

2.6.11 Preparation and storage of sera

Blood collected as described (section 2.6.10) was allowed to clot at room temperature and the clot released from the sides of the container using a glass rod. The clot was allowed to contract overnight at 4°C, the

serum transferred to a 10ml or 50ml Falcon tube and centrifuged at 3,000rpm for five minutes in the bench-top centrifuge at room temperature, to remove red blood cells. The serum was then snap-frozen in liquid nitrogen in several small, and one large, aliquots and stored at -70°C. Serum aliquots were thawed at room temperature when required and kept on ice until they were snap frozen again, prior to storage at -70°C.

2.6.12 Western blotting

Proteins were electrophoretically transferred from SDS-polyacrylamide gels to nitro-cellulose membranes using a modification of the method of Towbin et al (1979). Following SDS-PAGE (section 2.6.2) the gel was soaked in Western transfer buffer (20mM Tris, 192mM glycine, pH8.3) for thirty minutes at room temperature, together with four pieces of Whatman 3MM paper, two Scotchbrite pads and a piece of nitrocellulose membrane (BA85, Schleicher and Schuell; cut to dimensions slightly larger than the gel). These were placed in the cassette of a BioRad TransBlot apparatus in the following order: one Scotchbrite pad, two pieces of 3MM paper, the gel, the nitrocellulose membrane, two pieces of 3MM paper and finally the second Scotchbrite pad. Care was taken to remove any air bubbles between the different layers. The cassette was inserted into the TransBlot electrophoresis tank (with the gel oriented towards the anode), which contained Western transfer buffer. Cooling was supplied by a water cooling system. Following transfer (at 0.1A overnight or 0.3A for four hours), the proteins bound to the membrane were stained for one minute in Ponceau-S staining solution (Sigma) and the blot destained by washing the membrane with dH_2O. The membrane was then cut into strips, if necessary, and incubated at 37°C for one hour with gentle agitation in 30ml blocking solution (table 2.5). It was then transferred to a plastic heat-sealable bag and incubated with 5-20ml of primary antibody solution (1:500 to 1:1000 dilution of antiserum in the appropriate buffer, Table 2.5) at 4°C, overnight or at room temperature for two hours, with agitation. The membrane was washed in three changes of wash buffer (table 2.5), at room temperature for fifteen minutes each, with agitation, and was then incubated with either ^{125}I-protein A at 37°C or alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (table 2.5). It was then washed, as above, and exposed to

X-ray film (for ^{125}I-labelled protein A), or developed by placing in 5ml of freshly prepared alkaline phoshatase developer solution (NBT and BCIP supplied by Promega), and incubated until the bands reached the desired intensity. Development was stopped by washing the membrane in dH_2O, and the membrane was then dried in air, on filter paper and stored at room temperature in the dark.

Table 2.5: Solutions for Western blotting

	Detection System	
	^{125}I-protein A	Goat anti-rabbit IgG
Blocking Solution	PBS, 5% (w/v)BSA	TBS 5% (w/v) BSA
Primary Antibody Solution	PBS 1% (w/v) BSA	TBS 1% (w/v) BSA
Washing Solution	PBS	TBS
Development Solution	10μCi ^{125}I protein A, PBS, 1% (w/v) BSA	0.1MTris-HCl pH9.5, 0.125M NaCl, 10mM $MgCl_2$, 0.33mg/ml BCIP, 0.165 mg/ml NBT

2.6.13 Affinity purification of antibodies

Antibodies were purified using a modification of the method of Robinson et al (1988). A crude lysate of E.coli cells in which fusion protein synthesis had been induced was subjected to preparative SDS-PAGE and transferred to a nitrocellulose membrane (section 2.6.12). The membrane was stained with Ponceau-S solution (Sigma), and a horizontal strip of the membrane containing the fusion protein band was excised and blocked for

one hour at 37°C in TBS, 5% w/v BSA. The strip was then incubated with 15ml of a 1:300 dilution of antiserum in TBS, 1% (w/v) BSA, overnight at 4°C, or for two hours at room temperature, with agitation. The membrane strip was washed in 3 changes of TBS, at room temperature, for 15 minutes. Antibodies were eluted by incubating the strip at 4°C for four minutes in 1.5ml glycine elution buffer (0.1M glycine-HCl pH2.2, 50% (v/v) glycerol). The eluate was immediately neutralized with 75µl 1M Tris base, snap-frozen in liquid nitrogen and stored at -70°C.

2.6.14 Immunoprecipitations

2.6.14.1 High stringency immunoprecipitation from ^{35}S-labelled yeast extracts

Immunoprecipitation of ^{35}S-labelled proteins from yeast extracts was performed by a modification of the procedure of Lee et al (1986).

Proteins were radioactively labelled in vivo by adding 300-500µCi of L-^{35}S-methionine to 400ml cultures of yeast cells (starting O.D. 0.025 to 0.05) in YMM (supplemented according to strain and plasmid selection requirements) and incubated overnight at 30°C. When the O.D. of the culture had reached approximately 0.4., the cells were harvested and lysed by sonication (section 2.6.5).

The crude extract was diluted to 4ml with 1xPBS, 1% (v/v)NP40, 10% foetal calf serum, 1mM PMSF, and divided into 6-12 aliquots of equal volume. Polyclonal serum (5µl) was added to each aliquot and the tubes were incubated at 4°C overnight, with mixing. A 1:1 slurry (30µl)of swollen Protein A separose (PAS, Sigma) beads: NET buffer (50mM Tris-HCl pH7.5, 0.15M NaCl, 5mM EDTA) was then added. The mixture was incubated for one hour at 4°C with mixing and the PAS beads sedimented by centrifugation (ten seconds in the microcentrifuge at 4°C), The supernatant was removed and the PAS beads subjected to the following series of washes at room temperature, with mixing: 2x30 minutes in HEN buffer (0.1M HEPES-NaOH pH7.9, 1M NaCl, 1% (v/v)NP40, 10mM EDTA, 1mM PMSF); 2x30 minutes in LT buffer (0.5M LiCl, 0.1M Tris-HCl pH8.8, 0.1% (w/v) SDS, 1% (v/v) NP40); ten

minutes in HEN buffer. After the final wash the beads were resuspended in 15µl of NET buffer, 20µl of SDS-PAGE loading buffer (section 2.6.2) added, and heated to 100°C for five minutes. The whole suspension was loaded onto an SDS-polyacrylamide gel and electrophoresis was performed as described (section 2.6.2). Following Coomassie Blue staining (section 2.6.3) the gel was incubated for twenty minutes in Amplify (Amersham) at room temperature, with agitation, dried at 80°C under vacuum, and exposed to X-ray film .

2.6.14.2 Low stringency immunoprecipitation from yeast in vitro splicing extracts

This procedure is based on that described by Lossky et al (1987). PAS was swollen in NTN buffer (50mM Tris-HClpH7.5, 150mM NaCl, 0.1% (v/v) NP40) and washed twice with this buffer by resuspension and brief centrifugation in the microcentrifuge at room temperature. PAS beads (5-15µl packed volume) were then incubated for one hour, with mixing, at room temperature with 3-5µl of antiserum diluted in 100µl NTN buffer or with 15µg of anti-m$_3$G antibody in 100µl of NTN buffer, or with 200-400µl of anti-PRP2 antibodies, affinity purified as described (section 2.6.13). The PAS-IgG was sedimented by centrifugation as above and washed three times with 1ml of NTN buffer, as above. Yeast in vitro splicing reactions or mock splicing reactions,(without added pre-mRNA) were placed on ice, and an equal volume of cold Q buffer (400mM KCl, 2mM magnesium acetate, 20mM EDTA, 64mM Tris HCl pH7.5), plus 6mg of yeast total RNA per 10µl splicing reaction, were added. This mixture was added to the PAS-IgG and incubated for one to two hours at 4°C with agitation. The PAS-IgG was sedimented by centrifugation for ten seconds at 4°C in the microcentrifuge and was then washed three times with cold NTN buffer at 4°C. The immunoprecipitate was then treated for SDS-PAGE, or RNA extracted from it, as described below.

To extract RNA from the immunoprecipitate, the PAS beads were incubated with 20µl of proteinase K solution (50mM Tris-HCl pH7.5, 0.3M NaCl, 5mM EDTA, 1.5% (w/v) SDS, 2mg/ml proteinase K) for fifteen minutes at 37°C with agitation and 80µl of 150mM sodium acetate pH5.2, 20µg/ml tRNA added. The mixture was extracted twice with phenol-chloroform, and RNA precipitated

with ethanol. The RNA was then analysed by denaturing polyacrylamide gel electrophoresis (section 2.4.9).

2.6.15 In vitro translation

10µl reaction mixtures for in vitro translation contained: 65-70% (v/v) rabbit reticulocyte extract (Amersham, N150: nuclease treated, amino acid depleted), 10µl L-^{35}S-methionine, 227 mM potassium acetate, 2mM magnesium acetate, 50µm amino acids (except methionine), 0.5-2µl T7 RNA or control RNA (section 2.1.6.3). Reactions were incubated at 30°C for one hour and stopped by freezing. Reticulocyte extract was stored in 50µl aliquots in liquid nitrogen.

CHAPTER 3

Generation and Characterization of Anti-PRP2 Antisera

3.1 Construction of lacZ- and trpE-PRP2 Fusion Proteins

As a first step towards the characterization of PRP2 protein and its function, it was decided to raise antibodies to the protein. The approach chosen was to construct fusions between *E. coli* proteins and different regions of the PRP2 protein, and to inject these fusion proteins into rabbits.

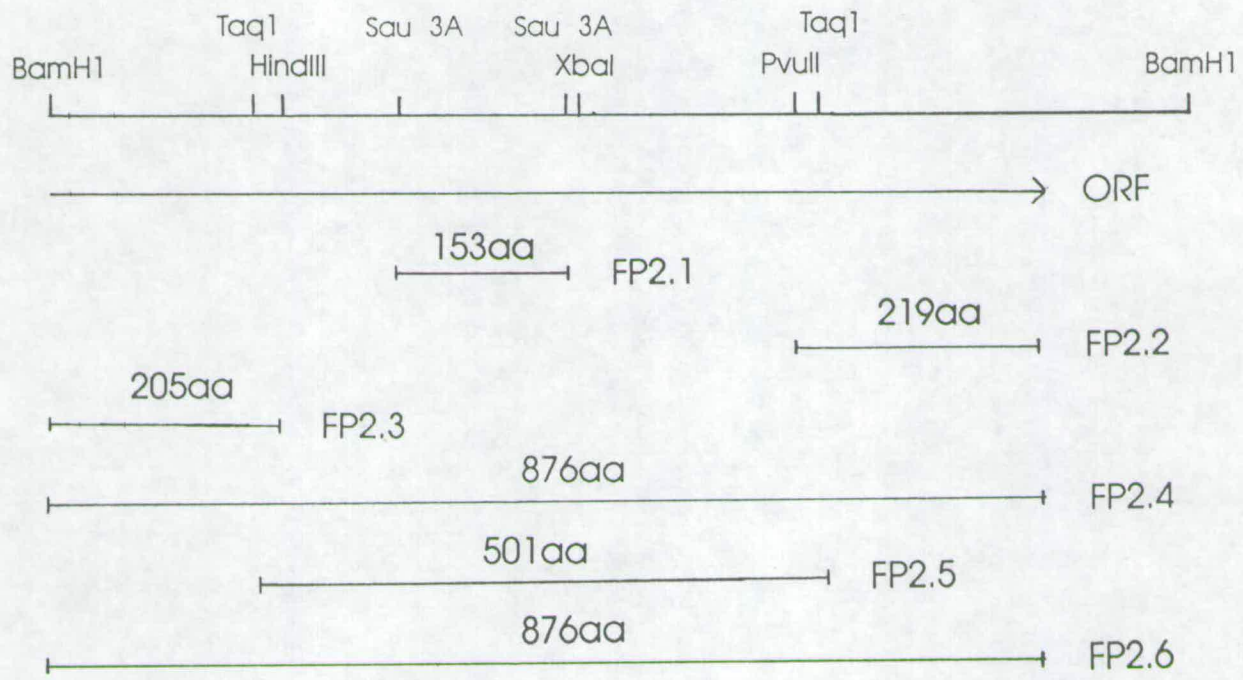
Two types of fusion proteins were constructed. The first type, which was used to raise anti-PRP2 antibodies, were fusions of different regions of PRP2 protein to the C-terminus of *E. coli* β-galactosidase. These constructs were based on a set of *E. coli* expression vectors, the pUR plasmids (Ruther and Muller-Hill,1982), which contain the whole of the *E. coli lacZ* gene and its IPTG-inducible promoter, upstream of a polylinker, into which the *PRP2* gene fragments were cloned. The polylinker is joined to the *lacZ* gene in a different reading frame in each vector and, knowing the sequence of the *PRP2* gene (J. Beggs, unpublished), it was therefore possible to select the appropriate vector, in order to produce an in-frame fusion between the *lacZ* gene and the different *PRP2* gene fragments.

The different fusion constructs are designated pFP2.1 to pFP2.6, in chronological order of construction, and are summarised in figure 3.1. In general, *E. coli* clones containing the desired construct were identified by screening transformants with radioactively labelled PRP2 probes, followed by restriction mapping of plasmids isolated from positive clones. These clones were then tested for production of appropriately sized fusion proteins by SDS-PAGE.

Figure 3.1 PRP2 fusion constructs

Top: restriction endonuclease map of the PRP2 gene, showing restriction sites used in construction of fusion plasmids. Not all TaqI and Sau3A sites are indicated. The BamHI site at the 5' end of the gene was introduced by cutting at an AflIII site and inserting BamHI linkers (see section 4.1.2). The open reading frame is indicated by the arrow marked ORF.

Bottom: the regions of PRP2 protein present in the different fusion proteins FP2.1-2.6 are indicated, with the number of amino acids in the PRP2 portion of the fusion protein. FP2.1-2.5 are fusions to the C-terminus of E. coli β-galactosidase; FP2.6 is a fusion to the C-terminus of a 33kD fragment of E. coli trpE protein.


Taq1
Sau 3A
Sau 3A
Taq1
BamH1
HindIII
XbaI
PvuII
BamH1
ORF
153aa
FP2.1
219aa
FP2.2
205aa
FP2.3
876aa
FP2.4
501aa
FP2.5
876aa
FP2.6

The first plasmid, pFP2.1 was constructed by M. Lee (Lee et al, 1986). pFP2.2 was constructed as follows (figure 3.2): pY2519+ (Table 2.3) was digested with PvuII, and BglII linkers were added. The DNA was then digested with XbaI and BglII and a 1.7kb fragment containing the 3' region of the PRP2 gene (see figure 3.1) was gel purified. This fragment was ligated to pUR288, cut with BamHI and XbaI, to create pFP2.2.

pFP2.3 (figure 3.3) was constructed by using a BamHI site, which was inserted directly before the ATG start codon of the PRP2 gene (see section 4.1.2). pBM-PRP2 (section 4.1.2) was digested with BamHI and HindIII, and a 0.6kb fragment from the 5' end of the gene was purified and ligated to pUR289, cut with BamHI and HindIII.

pFP2.4 (figure 3.3), containing the whole of the PRP2 gene was constructed by ligating a gel purified 3kb BamHI fragment (from pBM-PRP2) to pUR289, cut with BamHI.

pFP2.5 (figure 3.4), contains a 1.5kb TaqI fragment which encodes the central region of the PRP2 gene. This fragment was previously cloned into the polylinker region of M13mp19, by J.Beggs. It was cut out of this polylinker with BamHI and HindIII, and ligated to pUR291, cut with BamHI and HindIII.

The second type of PRP2 fusion protein produced was FP2.6, which contains the whole of the PRP2 protein fused to the C-terminus of a fragment of the E. coli trpE protein (Dieckmann and Tzagoloff, 1985). The vector used in this case, pATH1, contains the trpE gene, under the transcriptional control of the indoleacrylic acid (IAA)-inducible trp operon promoter. This vector is part of the pATH series, which have a polylinker at the 3' end of a fragment ofthe trpE gene, fused to the gene in different reading frames. pFP2.6 was constructed by ligating a purified 3kb BamHI fragment containing the PRP2 gene (see above, pFP2.4) to pATH1, cut with BamHI.

E. coli cells carrying the different fusion constructs were induced for fusion protein synthesis (section 2.6.6, 2.6.7), the cells lysed and the

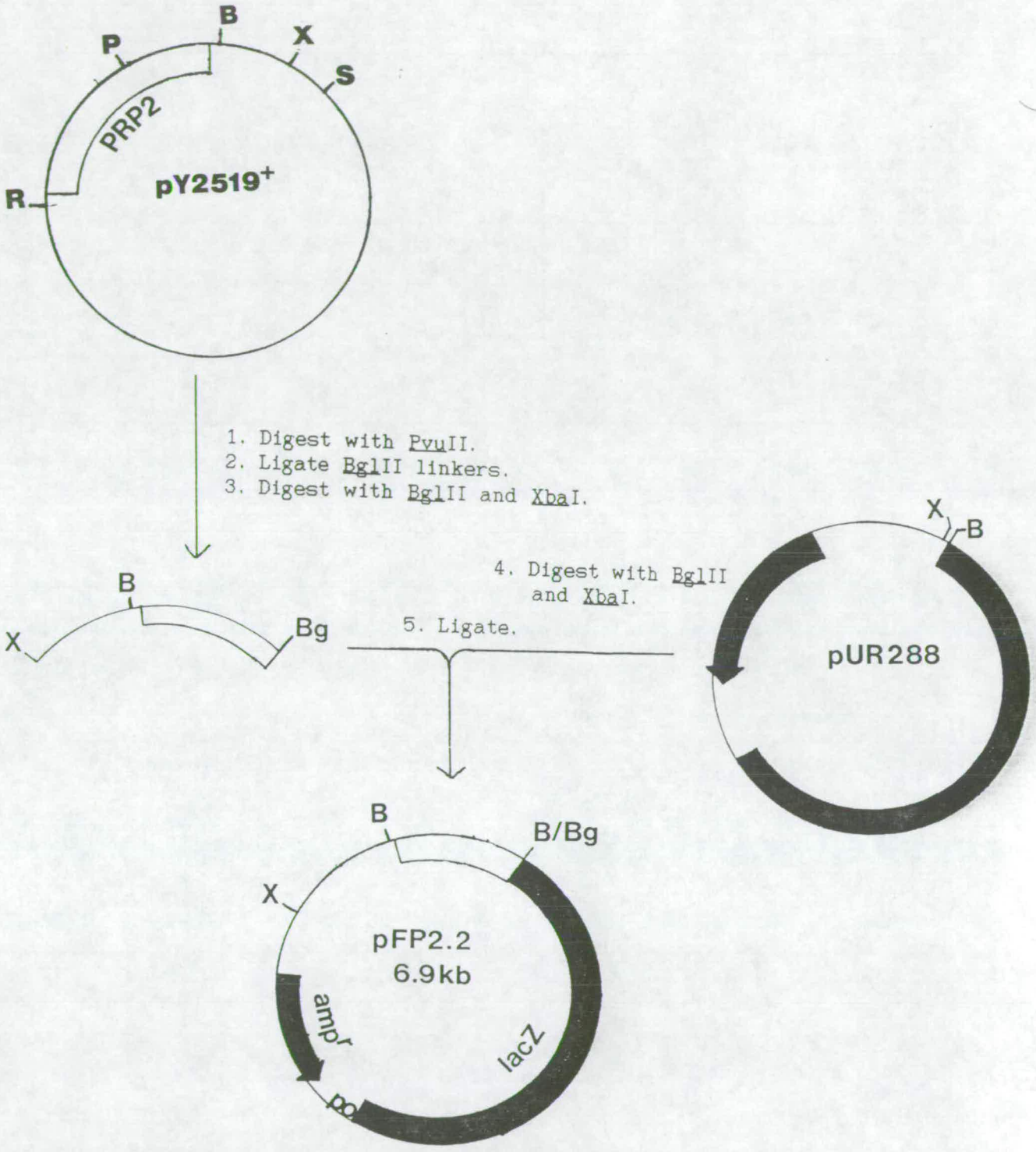


Figure 3.2 Construction of pFP2.2

Open boxes: PRP2 coding sequences. Closed boxes: vector genes. Restriction sites: R-EcoRI, P-PvuII, B-BamHI, X-XbaI, S-SalI, Bg-BglII. p-lac promoter, o-lac operator.

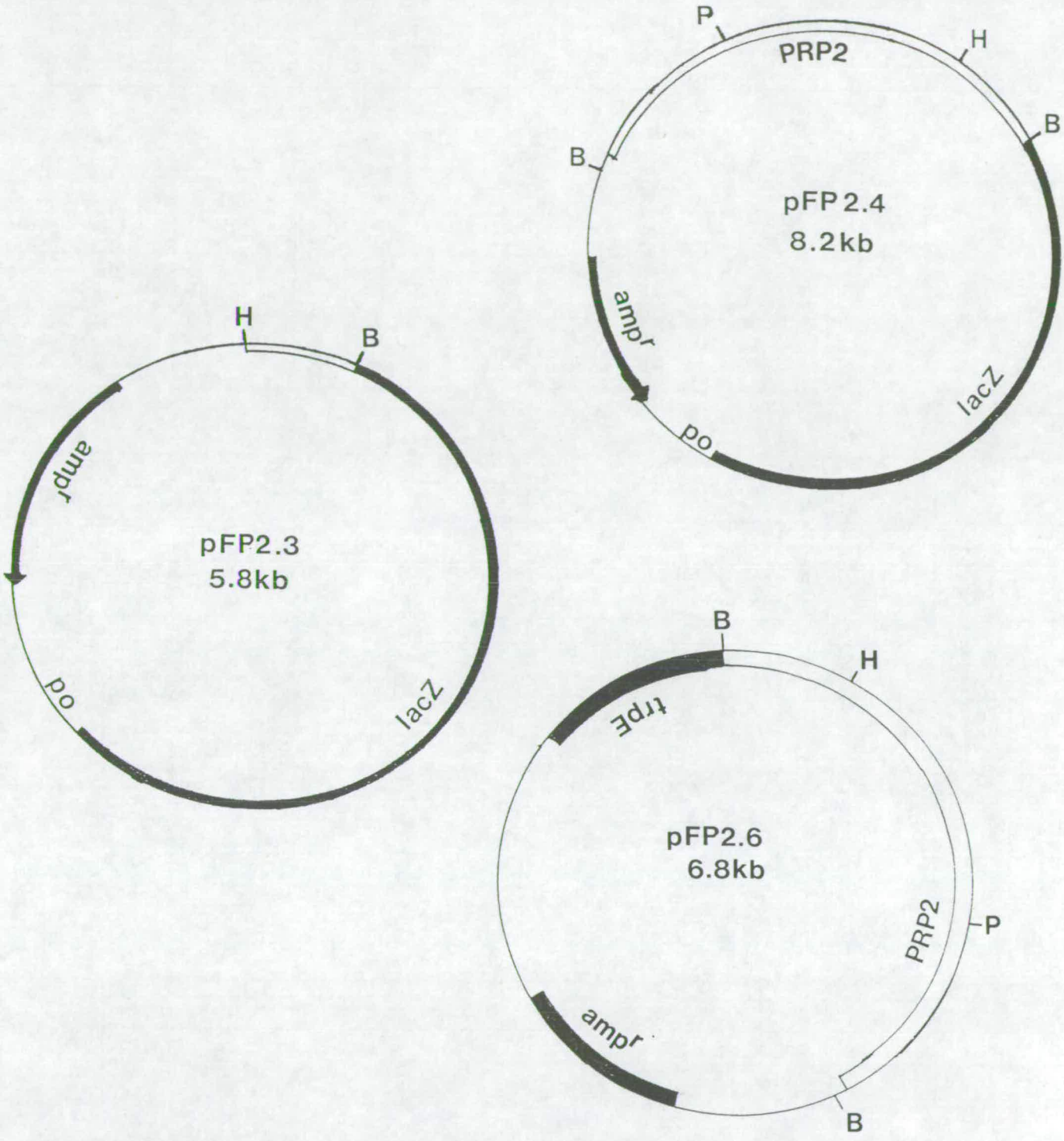
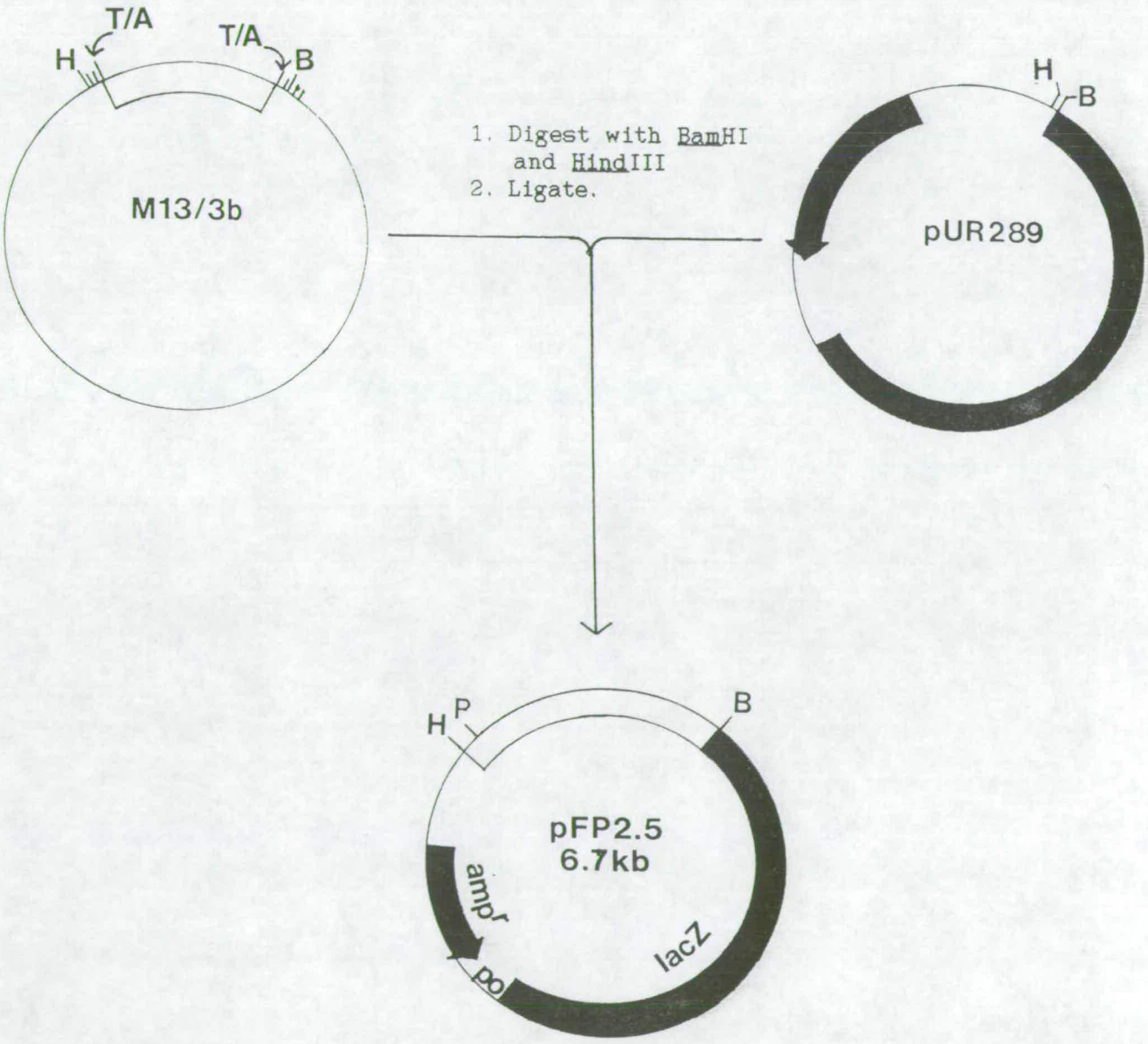


Figure 3.3 Plasmid maps of pFP2.3, pFP2.4 and pFP2.6

Open boxes: PRP2 coding sequences. Closed boxes: vector genes. Restriction sites: P-PvuII, B-BamHI, H-HindIII p-lac promoter, o-lac operator.

Figure 3.4 Construction of pFP2.5

Open boxes: PRP2 coding sequences. Closed boxes: vector genes. Restriction sites: B-BamHI, H-HindIII T-TaqI, A-AccI. p-lac promoter, o-lac operator.



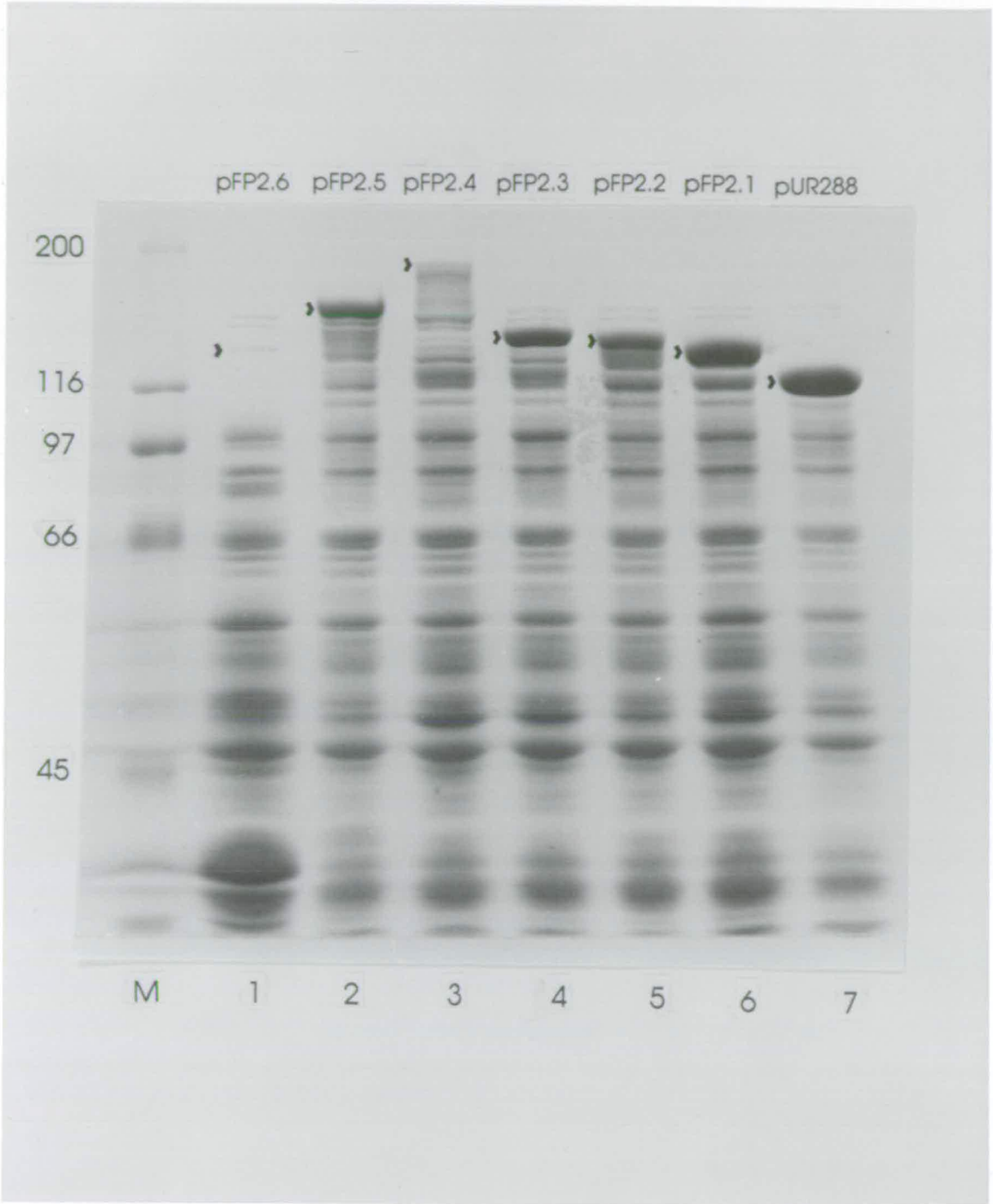
proteins produced analysed by SDS-PAGE, with the results shown in figure 3.5. In each track the band indicated by an arrow is the full sized fusion protein. The more stable fusion proteins are produced in large quantities, and are the strongest bands in the track, illustrating the efficient expression which can be achieved using these vectors. It is probable that different amounts of fusion proteins are produced in cells containing the different constructs because of different susceptibilities of the fusion proteins to proteolysis: FP2.2 is less stable than FP2.1, 2.3 and 2.5, and the two PRP2 fusions which contain the whole of the PRP2 protein, FP2.4 and FP2.6, are much less stable than FP2.2. In the case of FP2.6, large amounts of a protein of the same size as the trpE protein are produced (lane 1), clearly demonstrating the sensitivity of the PRP2 protein to proteolysis, when not protected by β-galactosidase. It is also possible that differences in efficiency of translation and/or stability of mRNAs coding for the various fusion proteins account for part of the differences in quantity of fusion protein produced.

The apparent molecular weights of the fusion proteins are approximately as expected from the lengths of PRP2 coding sequence which are fused to the lacZ gene. There are, however, two unexpected results. The first is that despite the fact that FP2.2 contains more PRP2 coding sequence than FP2.3 (219 vs 205 amino acids) the apparent molecular weight of FP2.3 is slightly greater than that of pFP2.2. (These constructions have not been sequenced, but are correct according to Southern blotting, using PRP2 probes; not shown). There is an in-frame stop codon in the pUR289 sequence, immediately downstream of the polylinker, ruling out the possibility that the size of FP2.3 is due to translational readthrough into vector sequences.

There are two possible explanations of this discrepancy. The first is simply that pFP2.3 migrates anomalously slowly in SDS-polyacrylamide gels. This has been observed for a number of proteins, including β-galactosidase fusion proteins (G. Anderson personal communication). There are no obvious sequences in FP2.3 which have been shown to cause such effects in other proteins. The second explanation relates to the second unexpected result in figure 2: although the expected molecular weight of pFP2.4 is approximately 216 kilodaltons, its apparent molecular weight is slightly

Figure 3.5 PRP2 fusion proteins

E. coli strain BMH71-18 carrying pUR288or pFP2.1 to pFP2.5, and HB101 carrying pFP2.6, were induced for fusion protein synthesis, the cells lysed by heating at 100°C for 5 minutes in SDS-PAGE sample buffer,and the proteins produced analysed by electrophoresis on an 8.5% SDS-polyacrylamide gel. The gel was stained with Coomassie blue, following electrophoresis. The plasmid carried by the cells is indicated at the top of each lane. In each lane the band of full sized fusion protein (or, in lane 7, β-galactosidase) is indicated by an arrow. Lane M: marker proteins, with sizes indicated in kD.


pFP2.6
pFP2.5
pFP2.4
pFP2.3
pFP2.2
pFP2.1
pUR288
200
116
97
66
45
M
1
2
3
4
5
6
7

less than 200kD. Using more sensitive western blotting techniques, it has not been possible to detect any β-galactosidase-containing fusion proteins of higher molecular weight in whole cell extracts made from cells carrying pFP2.4 (not shown). It is possible that the C-terminal region of PRP2 protein, which is common to FP2.2 and FP2.4 is extremely susceptible to protease degradation in E. coli, and is very rapidly degraded, resulting in a lower apparent molecular weight.

3.2 Production of PRP2-Specific Antisera

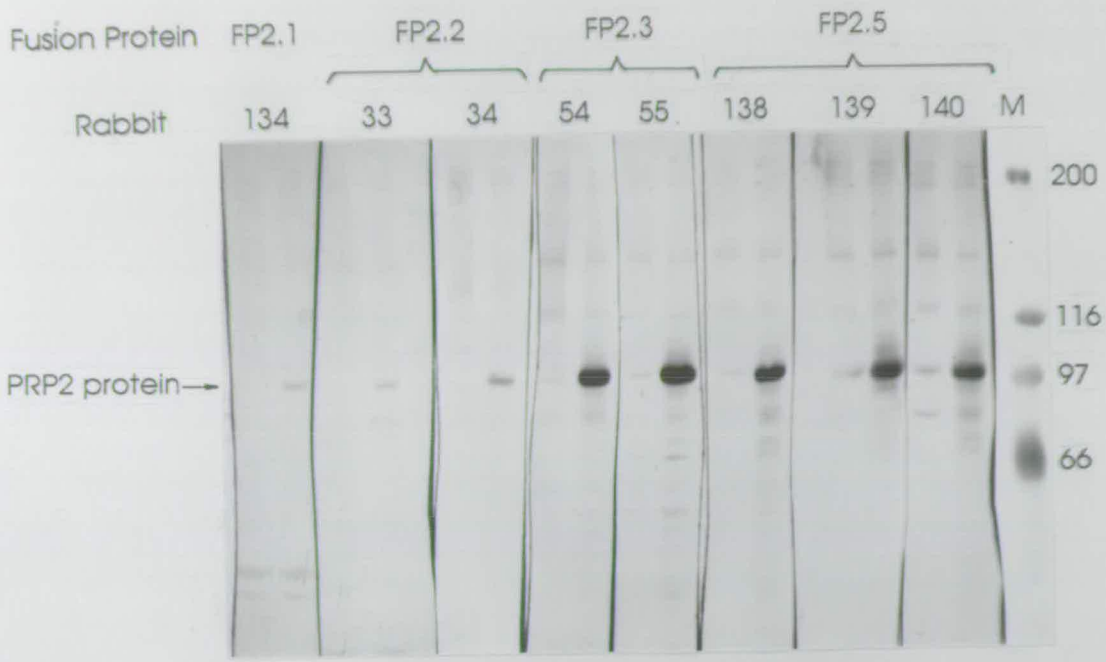
Fusion proteins were purified for for use as antigens by two different methods (section 2.6.9). The first method, preparative SDS-PAGE, produced tens of micrograms of essentially pure, full size fusion proteins. The second method, urea fractionation resulted in hundreds of micrograms of protein, which was mainly β-galactosidase fusion protein. Proteins produced by the second method are partly native .

Each fusion protein was injected into either two or three rabbits. This helped to increase the likelihood of obtaining sera with a high titre of antibodies, since the titre varied considerably between different rabbits. It also helped to identify immunological artefacts peculiar to particular animals. Injections were continued, at intervals of 3 weeks to 1 month, until sera which reacted well with PRP2 protein on western blots were obtained (see below) whereupon the animals were given one further injection and then sacrificed. This normally occurred after three or four injections. However, in the case of FP2.2 seven injections were necessary.

A comparison of the reaction of the different antisera with the PRP2 protein is shown in figure 3.6. Yeast cell extracts were made by the sonication method (section 2.6.5) from two different strains of yeast (see below). The extracts were analysed by SDS-PAGE followed by Western blotting, and the blot probed with the different antisera. Each strip of the blot contains two tracks: the left hand track is extract derived from strain DJY40/pKV49 and the right hand track is from DJY40/pKV-PRP2

Figure 3.6 Comparison of antisera raised against different PRP2 fusion proteins.

A Western blot of an 8.5% SDS polyacrylamide gel was cut into strips and probed with a 1:1000 dilution of the different antisera, in an equal volume of buffer. The blot was developed using the alkaline phosphatase procedure. Each strip consists of two lanes; the left hand lane contains 10μl of extract, made by sonication, from DJY40 cells carrying pKV49; the right hand lane contains 10μl of extract from DJY40 cells carrying pKV-PRP2 (see text). The number of the rabbit from which the antiserum was taken is indicated at the top of each strip, as is the fusion protein with which each rabbit was injected. Lane M: marker proteins, with sizes indicated in kD.


Fusion Protein
FP2.1
FP2.2
FP2.3
FP2.5
Rabbit
134
33
34
54
55
138
139
140
M
200
116
97
66
PRP2 protein

(section 4.1.2). These differ in that cells carrying pKV-PRP2 produce high levels of PRP2 protein, when grown in media which contain galactose as carbon source, while cells carrying the parent vector, pKV49, produce normal levels of PRP2 protein, from the chromosomal gene. On each strip, as expected, the right hand track has a higher signal at the expected size of PRP2 protein (approximately 100kD), which facilitates identification of PRP2 protein.

The titre of antibodies against PRP2 protein varies considerably between the different sera, although all show some reaction with the protein. There also exist smaller differences between different rabbits injected with the same fusion protein (cf rabbits 33 and 34). The sera raised against fusion proteins 2.3 and 2.5 have a far higher titre of anti-PRP2 antibodies than those raised against FP2.1 or FP2.2. This might be due to the fact that the former fusion proteins were prepared by the urea fractionation procedure, whereas the latter were produced by gel purification. However, further attempts to produce antisera to FP2.2, using the urea fractionation procedure to prepare the antigen, have not been successful, and as mentioned above it took a larger number of injections of FP2.2 than of other fusion proteins to obtain a response to PRP2. It is possible that the C-terminal region of PRP2 protein is not strongly immunogenic in rabbits.

All the sera show some reactions with other yeast proteins, such as the triplet of bands at approximately 200kd. These bands have been observed on western blots using rabbit antisera raised against different (non-PRP2) fusions and are partly due to cross-reactions of both the primary and the secondary antibodies with these proteins (M. Lossky, G. Anderson, personal communication). The smear running ahead of the PRP2 protein in the right hand tracks of the strips of the blot probed with sera 54, 55, 138, 139 and 140, is almost certainly due to breakdown products of the PRP2 protein.

3.3 Immunoprecipitations With Anti-PRP2 Antisera

All the anti-PRP2 antisera have been used to immunoprecipitate the PRP2 protein from yeast soluble protein extracts. An example of such an

Figure 3.7 Immunoprecipitations with anti-PRP2 antisera.

A. Immunoprecipitations with αFP2.1, αFP2.2 and α-peptide serum.

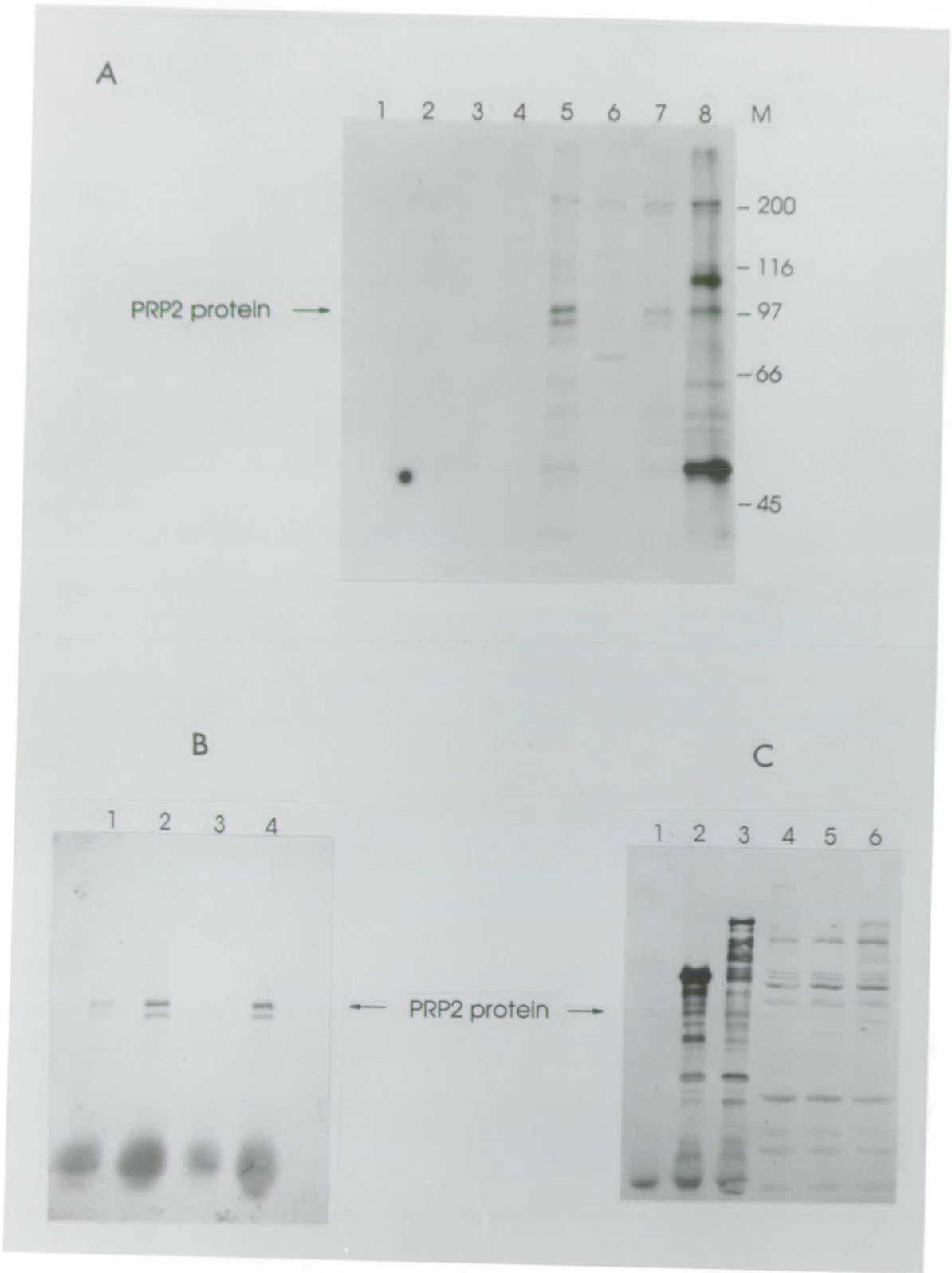
Immunoprecipitations were performed upon ^{35}S-labelled yeast soluble protein extracts, made by sonication, using 5μl of the different antisera and the immunoprecipitates analysed by electrophoresis on an 8.5% SDS-polyacrylamide gel. Following electrophoresis, the gel was fluorographed and exposed to X-ray film.

Lanes 1 and 2: immunoprecipitations from extract of KY117/pBM125 cells. Lanes 3 to 8: immunoprecipitations from extract of KY117/pBM-PRP2 cells (see text). Immunoprecipitations were with preimmune serum from rabbit 34 (lanes 1 and 3), immune serum from rabbit 134 (αFP2.1; lanes 2 and 7), immune serum from rabbit 34 (αFP2.2; lane5) immune serum from rabbit 33 (αFP2.2; lane4), immune serum from rabbit 122 (α-peptide serum, see text; lane 6). Lane 8 is identical to lane 7 except that 10μg of purified FP2.1 was added to the immunoprecipitation at the same time as the serum. Lane M: marker proteins, with sizes indicated in kD.

B. Immunoprecipitations with αFP2.3 and αFP2.5 antisera.

Immunoprecipitations (using 5μl of serum) were performed upon a 20μl 'mock' splicing reaction, (identical to a normal *in vitro* splicing reaction, but with no added pre-mRNA, using splicing extract made from BJ2412 cells). The immunoprecipitates were analysed by electrophoresis on an 8.5% SDS-polyacrylamide gel, followed by western blotting. The blot was probed with rabbit 139 serum (αFP2.5) and developed by the alkaline phosphatase procedure.

Lanes 1 and 2: immunoprecipitation from 'mock' splicing reaction with no added ATP in incubation prior to immunoprecipitation. Lanes 3 and 4: immunoprecipitation from mock splicing reaction with 2mM ATP (final concentration) added to incubation prior to immunoprecipitation. Immunoprecipitations were with serum 55 (αFP2.3; lanes 1 and 3) or serum 139 (αFP2.5; lanes 2 and 4).


A
1 2 3 4 5 6 7 8 M
PRP2 protein
200
116
97
66
45
B
1 2 3 4
PRP2 protein
C
1 2 3 4 5 6

experiment is shown in figure 3.7A. Extracts were made from two different yeast strains: KY117/pBM125 and KY117/pBM-PRP2. The latter carries the whole of the PRP2 gene under control of the GAL1 promoter, on a single copy yeast plasmid (section 4.1.2). This strain moderately overproduces the PRP2 protein when cells are grown on galactose as carbon source, compared to KY117/pBM125 which carries the parent vector. Yeast proteins were labelled *in vivo* with ^{35}S-methionine, and cells were lysed by sonication in a buffer containing 1% NP40. The extract was divided into equal aliquots and immunoprecipitations, with different antisera were performed, using stringent washing procedures (as described in section 2.6.14.1). The immunoprecipitates were analysed by SDS-PAGE, followed by fluorography.

Neither pre-immune serum, nor immune serum from rabbit 134 (αFP2.1) precipitate detectable amounts of PRP2 protein from KY117/pBM125 extracts (figure 3.7A, lanes 1 and 2). However, both 134 (lane 7) and 34 (αFP2.2; lane 5) immune sera precipitate significant amounts of PRP2 protein from KY117/pBM-PRP2 extracts. Serum from rabbit 33 (lane 6) is considerably less effective than that from rabbit 34 in immunoprecipitating PRP2 protein despite the relatively small difference between the two sera in detection of PRP2 protein on western blots (figure 3.6). This is presumably due to a higher titre, in the serum of rabbit 34, of antibodies against epitopes revealed in native PRP2 protein. The other antiserum used in this experiment was raised against a synthetic peptide from a region close to the N-terminus of PRP2 protein. This serum has been shown to immunoprecipitate PRP2 protein from yeast extracts (Lee *et al*, 1986), but in the experiment shown did not detectably immunoprecipitate the protein.

Figure 5.7B shows immunoprecipitations with sera from rabbits 55 and 139. In this case, immunoprecipitations were performed upon *in vitro* splicing extracts, made from cells which do not overproduce PRP2 protein. The extracts were incubated for 20 minutes under 'mock' splicing conditions (identical to those of an *in vitro* splicing reaction, but with no added pre-mRNA) in the presence or absence of added ATP (2mM). The reactions were then stopped, and immunoprecipitations performed using low stringency washing procedures, (section 2.6.14.2). PRP2 protein was detected in the immunoprecipitates by Western blotting. PRP2 protein appears as two

bands in this experiment, due, presumably, to degradation of the full size protein: a similar effect is observed in figure 3.7A, lane 5. Although sera 55 and 139 appear to react with PRP2 protein on Western blots with approximately equal efficiency, serum 139 is considerably more efficient at immunoprecipitating PRP2 protein from splicing extracts. It is possible that there is a higher titre of antibodies which recognise epitopes present in native PRP2 protein in serum 139 than in serum 55.

Although figures 3.7A and 3.7B are not strictly comparable, due to the different extracts and detection methods used, the fact that 55 and 139 sera detected PRP2 in extracts from cells that do not overproduce PRP2 protein suggests that they immunoprecipitate PRP2 protein more efficiently than 34 or 134 sera. This is expected from the stronger reaction of these sera with PRP2 protein on Western blots. Figure 3.7B also demonstrates that the efficiency of precipitation of PRP2 protein is not affected by the presence of ATP, in the incubation prior to immunoprecipitation (cf lanes 2 and 4).

A number of other labelled proteins are present in the immunoprecipitates shown in figure 3.7A. The amounts of these proteins, (of molecular weights 200kD, 58-62kD and 48-52kD), present in the immunoprecipitates does not correlate with the amount of PRP2 protein immunoprecipitated by the different antisera, and is in general variable between experiments. The proteins of molecular weight 200kD are almost certainly identical to the proteins of similar molecular weight, which are detected on western blots (figure 3.6) and which are known not to be related to PRP2 protein. It is probably that the bands observed in figure 3.7A are proteins which bind IgG non-specifically, probably via hydrophobic interactions. In pre-immune sera, which contain less IgG than immune sera, these bands are reduced. Repeated attempts to detect proteins which co-immunoprecipitate with PRP2 protein have failed to identify any such proteins.

In figure 3.7A, lane 8, a large excess of FP2.1 was added to the immunoprecipitation at the same point as the antiserum. This excess of fusion protein would be expected to compete with PRP2 protein for the

antibody, and thus reduce the intensity of the 100kD band. In fact, the intensity of the 100kD band is slightly increased in lane 8. The failure of FP2.1 to compete with PRP2 protein initially led to doubts that the 100kD band observed in immunoprecipitates was the PRP2 protein. The clue to understanding this result is the increased precipitation of 52, 112 and 200kD proteins in this lane; in some experiments the 100kD band was also strongly increased in intensity. In an immunoprecipitation of <u>in vitro</u> translated PRP2 protein, (i.e. where the PRP2 protein was the only ^{35}S - labelled protein present, section 4.3)in the presence of a β-galactosidase-PRP8 fusion protein, a similar effect was observed (not shown), indicating that PRP2 protein has a tendency to precipitate more strongly under these conditions. Thus, when partially denatured PRP2 fusion proteins are added to an immunoprecipitation, two processes compete: (i) competition of the fusion protein for anti-PRP2 antibodies and (ii) hydrophobic interactions between the fusion protein and PRP2 protein, followed by immuno-precipitation of PRP2 protein-fusion protein complexes by anti-β-galactosidase antibodies. In many cases the latter process is more efficient than the former, resulting in increased immunoprecipitation of PRP2 protein in the present of the competing fusion protein.

Another example of an antigen competition experiment is shown in figure 3.7C. Here, the antiserum used was from rabbit 139 and the immunoprecipitation performed on splicing extract, using low stringency washing procedures (section 2.6.14.2). Competition was performed by adding β-galactosidase (lanes 2 and 5) or FP2.5 (lanes 3 and 6) to the incubation of antiserum with PAS, prior to immunoprecipitation. PRP2 protein is just visible in lane 1, but due to the background of fusion protein and β-galactosidase breakdown products it is not possible to observe PRP2 protein in lanes 2 and 3. However, in the immuno-precipitation supernatants PRP2 protein is clearly visible in lane 6, but not in lanes 4 and 5, indicating that FP2.5, but not β-galactosidase competes with PRP2 protein for binding of anti-PRP2 antibodies. It is not clear why the hydrophobic interaction effect is not operative in this experiment, but it is possibly due to the fact that PRP2 protein is not over-expressed in splicing extract. If PRP2 protein is in a complex with other proteins, when it is expressed at a much higher level than the other

proteins in the complex, then the excess PRP2 protein will have exposed hydrophobic patches on its surface, which will tend to make it stick non-specifically to hydrophobic patches on the surface of partially denatured fusion proteins. Evidence from genetic studies (Last et al,1987) and from biochemical fractionation (Lin et al, 1987) suggests that PRP2 protein may be part of such a complex.

CHAPTER 4

Expression and Partial Purification of PRP2 Protein

There were two main reasons for creating *in vivo* and *in vitro* systems for the over-expression of PRP2 protein. The first was illustrated in chapter 3: over-expression as a means of identification of the protein. The second reason was to obtain an enriched source of PRP2 protein for partial purification (see section 4.5). In the course of this work, an assay system for PRP2 activity was developed, based on the system of Lustig *et al* (1986).

4.1 Over-expression of PRP2 Protein in Yeast

4.1.1 Constitutive expression of PRP2 protein using a high copy number vector

The plasmid pJDB2076, which was used to obtain over-expression of PRP2 protein under the control of its own promoter, contained the whole of the *PRP2* gene, which was sub-cloned into a high copy number yeast vector, pJDB207 (Lee *et al*, 1984; figure 4.1). A similar construct was used to obtain over-expression of PRP2 protein by Last *et al* (1986).

An example of the over-expression obtainable using this construct is shown in figure 4.2A. DBY747 cells, carrying pJDB207 or pJDB2076 were grown on yeast minimal medium, under plasmid selective conditions, and were lysed by sonication. The soluble protein extracts were analysed by Western blotting, and the blot probed with αFP2.1 serum (rabbit 134), or with pre-immune serum from the same rabbit. PRP2 protein was detected only in extracts from cells which carried pJDB2076 (figure 4.2A, lane 4). It is not possible to estimate accurately the degree of over-expression of PRP2 protein in this experiment since it was not detected in the control extract. However, the level of expression of PRP2 protein in cells carrying pJDB2076 is at least 30-fold higher than the control.

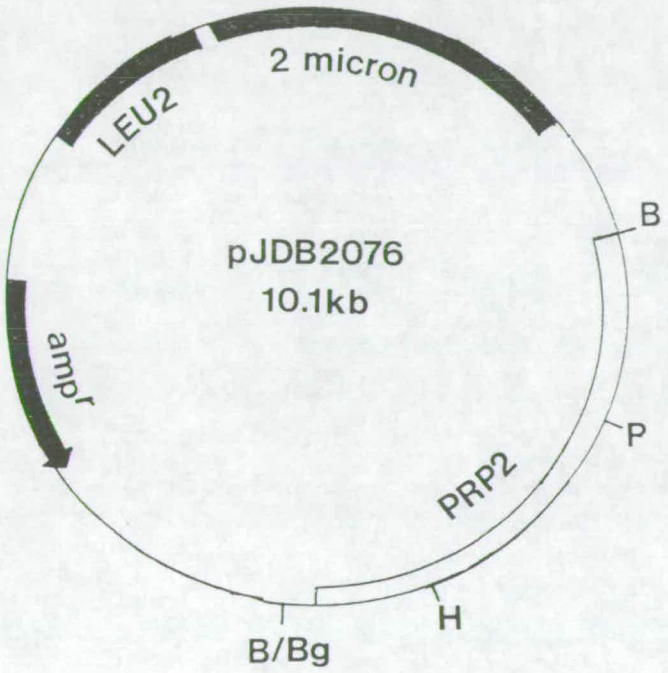


Figure 4.1 Plasmid map of pJDB2076

Open boxes: PRP2 coding sequences. Closed boxes: vector genes.
Restriction sites: P-PvuII, B-BamHI, H-HindIII, Bg-BglII.

Figure 4.2 Over-expression of PRP2 protein in yeast.

A. Over-expression of PRP2 protein in yeast using a high copy number vector.

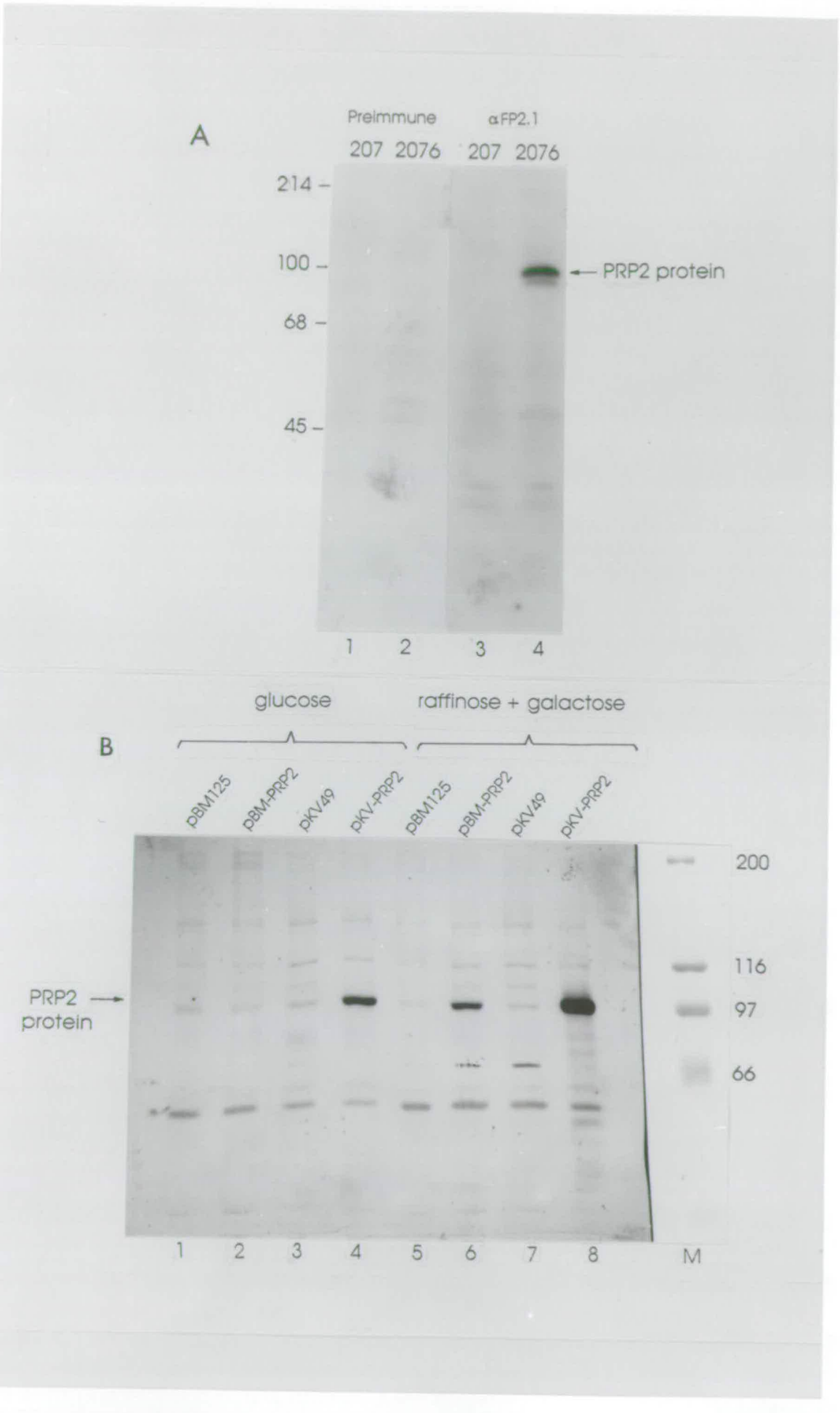
Extracts, made by sonication, of DBY747 cells carrying pJDB207 or pJDB2076, were analysed by electrophoresis on an 8.5% SDS-polyacrylamide gel, followed by Western blotting. The blot was cut into 2 strips and probed with either immune or pre-immune serum from rabbit 134 (αFP2.1) and developed using the ^{125}I-protein A procedure.

Lanes 1 and 3: extracts from cells carrying pJDB207. Lanes 2 and 4: extracts from cells carrying pJDB2076. Lanes 1 and 2: blot probed with 1:1000 dilution of preimmune serum from rabbit 134. Lanes 3 and 4: blot probed with 1:1000 dilution of immune serum from rabbit 134 (αFP2.1). Lane M: marker proteins with sizes indication in kD.

B. Over-expression of PRP2 protein in yeast under the control of the GALI UAS.

KY117 cells (carrying pBM125 or pBM-PRP2) and DJY40 cells (carrying pKV49 or pKV-PRP2) were grown on yeast minimal medium, with either glucose or raffinose plus galactose as carbon source. Extracts of cells, made by sonication, were analysed by electrophoresis on an 8.5% SDS-polyacrylamide gel, followed by Western blotting. The blot was probed with rabbit 55 antiserum (αFP2.3) and developed by the alkaline phosphatase procedure.

Lanes 1 to 4: cells grown with glucose as carbon source. Lanes 5 to 8: cells grown with raffinose plus galactose as carbon source. The plasmid carried by the cells from which extracts were made is indicated at the top of each lane. Lane M: marker proteins, with sizes indicated in kD.


A
Preimmune
αFP2.1
207 2076 207 2076
214 –
100 –
68 –
45 –
PRP2 protein
1 2 3 4
B
glucose
raffinose + galactose
pBM125
pBM-PRP2
pKV49
pKV-PRP2
pBM125
pBM-PRP2
pKV49
pKV-PRP2
PRP2 protein
200
116
97
66
1 2 3 4 5 6 7 8 M

Although extracts from cells carrying pJDB2076 consistently showed higher levels of expression of PRP2 protein than control extracts, this level was variable, and seemed to decrease with increasing time of storage of transformants on agar plates. Furthermore, pJDB2076 transformants often grew extremely slowly, relative to pJDB207 transformants. This suggests that high levels of PRP2 protein are inhibitory to growth in yeast, and that as a result, cells which have eliminated the plasmid, or in which the PRP2 gene on the plasmid was inactivated by recombination, are selected during growth in culture. Other laboratories which have used high copy number yeast plasmids containing the PRP2 gene have not reported similar difficulties, however; the reason for this discrepancy is not known.

In this laboratory (this work, and M. Lee, J.D. Beggs unpublished), the lack of reliable over-expression and growth of pJDB2076 transformants made this plasmid unsuitable for further experiments.

4.1.2 Regulated expression of PRP2 protein

In cells carrying pJDB2076, expression of the plasmid PRP2 gene is constitutive. Since it appeared to be the case that such constitutive production of high levels of PRP2 protein is deleterious to cell growth, it was decided to use an inducible/repressible expression system. With such a system, cells carrying the expression construct can be grown to suitable optical density under conditions which repress expression of PRP2 protein, (and which, therefore, are not deleterious to growth), and expression then induced by a change in growth conditions.

The yeast GAL1 promoter was chosen for controlled expression of the PRP2 gene. This promoter controls the transcription of the GAL1 gene according to growth conditions: when cells are grown using glucose as carbon source, transcription from the GAL1 promoter is repressed; when galactose is the carbon source, high levels of transcription are induced. Raffinose is a neutral carbon source, which neither represses nor induces transcription from this promoter.

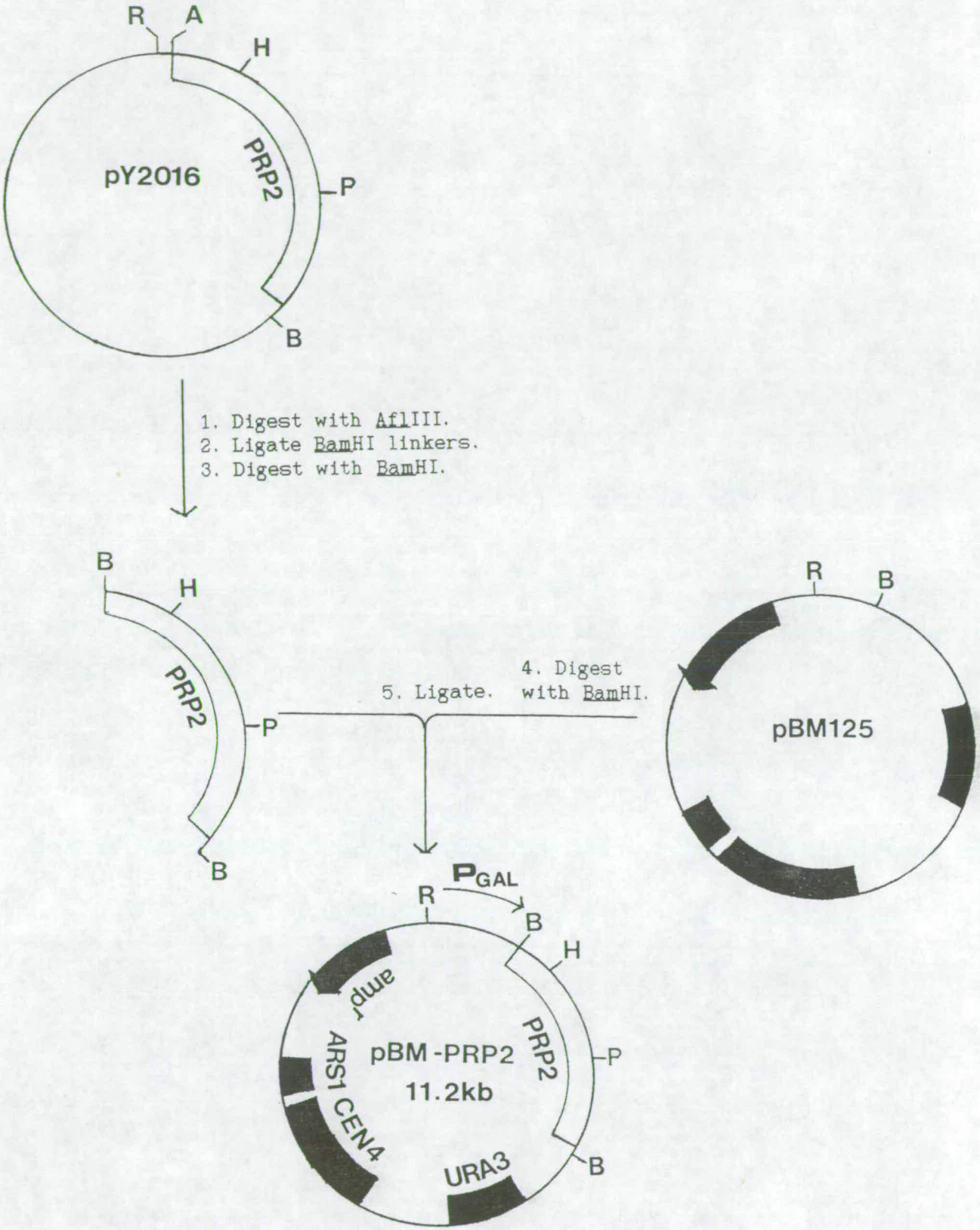


Figure 4.3 Construction of pBM-PRP2

Open boxes: PRP2 coding sequences. Closed boxes: vector genes.
Restriction sites: R-EcoRI, P-PvuII, B-BamHI, H-HindIII, A-AflIII.
P_{GAL}-GAL1 promoter.

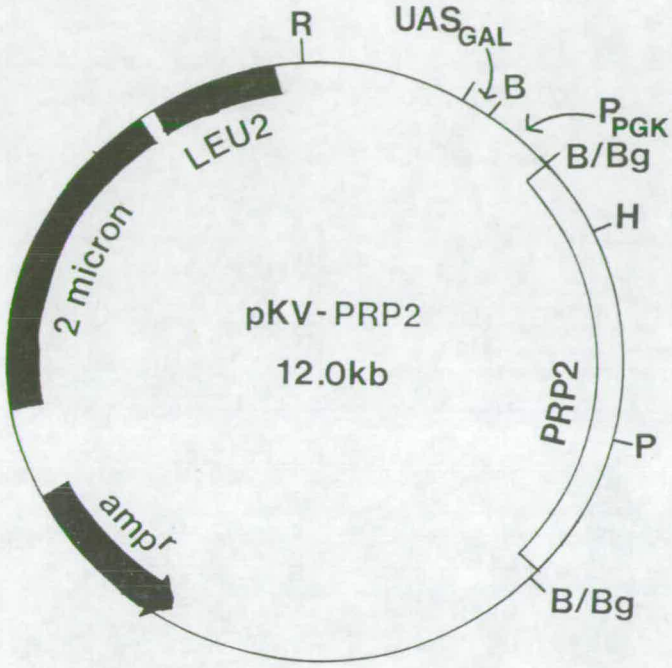


Figure 4.4 Plasmid map of pKV-PRP2

Open boxes: PRP2 coding sequences. Closed boxes: vector genes. Restriction sites: R-EcoRI, P-PvuII, B-BamHI, Bg-BglII, H-HindIII. UAS_{GAL}-GAL1 upstream activator sequence, P_{PGK}-PGK promoter.

In order to place the PRP2 gene under control of the GAL1 promoter, it was necessary to separate the PRP2 gene from its own promoter. (Attempts to obtain over-expression by placing the PRP2 promoter under control of the GAL1 upstream activator sequence (UAS), on a low copy number plasmid (constructed by M. Lossky), did not produce detectably increased amounts of PRP2 protein, as assayed by Western blotting; not shown). In order to do this the presence of an AflIII site at the methionine initiator codon of the PRP2 gene was exploited (figure 4.3). Plasmid pY2016 (Lee et al, 1984) was cut with AflIII, and overhanging 5' ends filled in with Klenow enzyme. BamHI linkers were then added, the DNA digested with BamHI, and a 3kb DNA fragment containing the PRP2 gene purified. This fragment was ligated to pBM125, cut with BamHI, thus inserting the PRP2 gene downstream of the GAL1 promoter. The BamHI site inserted at the start of the PRP2 gene was utilized in a number of other constructs (e.g. figure 3.1).

In order to combine the advantages of the GAL1 induction/repression system with those of a very strong promoter, and high plasmid copy number, the vector pKV49 was utilized. This plasmid contains the yeast phosphoglycerate kinase promoter, under the control of the GAL1 UAS, and is maintained at high copy number.

In order to insert the PRP2 gene into this plasmid, pBM-PRP2 was cut with BamHI and the 3kb PRP2 fragment was gel purified. This fragment was ligated to pKV49, cut with BglII, creating pKV-PRP2 (figure 4.4).

pBM-PRP2. PKV-PRP2, and their respective parent vectors were transformed into yeast strains with the appropriate selectable markers. The control of expression of PRP2 protein in cells carrying these plasmids was examined by Western blotting of protein extracts, produced by sonication (figure 4.2B). Cells (KY117 carrying pBM125 or pBM-PRP2, DJY40 carrying pKV49 or pKV-PRP2) were grown overnight in yeast minimal medium (YMM) plus 2% glucose in order to repress expression of PRP2 protein, or on YMM plus 2% raffinose and 2% galactose to induce expression (section 2.6.8). Raffinose is present in the latter case as an additional, neutral, carbon source, since the yeast strains used grow more slowly on galactose alone than on glucose.

As figure 4.2B, lanes 2 and 6 indicate, cells carrying pBM-PRP2 over-express PRP2 protein only when grown on galactose. Cells carrying the parent vector pBM125 do not over-express PRP2 protein under either set of growth conditions (lanes 1 and 5). pBM-PRP2-carrying cells produce approximately ten times as much PRP2 protein when grown on raffinose plus galactose than when grown on glucose as carbon source.

For cells carrying pKV-PRP2 the situation is slightly more complex, since PRP2 protein is over-expressed even when cells are grown on glucose (lane 4). It appears that with this plasmid, glucose repression is not fully operative. One possible explanation of this effect is that it is due to the high copy number of pKV-PRP2. The large number of copies of the GAL1 UAS may titrate out the GAL80 protein, which represses transcription when cells are grown on glucose, thereby allowing transcription from the strong PGK promoter.

Using a more sensitive detection system, a similar phenomenon was observed with pBM-PRP2-carrying cells. Thus, when pBM125 and pBM-PRP2 were transformed into a temperative sensitive prp2-1 strain (JBY27), and the transformants were grown at the restrictive temperative, as expected, pBM-PRP2, but not pBM125, was able to complement the temperature sensitive growth defect. This complementation occurred when cells were grown on either glucose, or on raffinose plus galactose as carbon source (data not shown) indicating that there was a small amount of transcription from the GAL1 promoter, even when the cells were grown on glucose.

When cells carrying pKV-PRP2 are grown on raffinose plus galactose, PRP2 protein is produced at extremely high levels (100-200 times background levels), demonstrating that the PGK promoter is under the control of the GAL UAS. It was possible to visualize the PRP2 protein by Coomassie Blue staining of a replica of the gel shown in figure 4.2B: a band of PRP2 protein was only visible in lane 8 (not shown). This experiment indicates that expression of PRP2 protein using the GAL regulation system was both efficient and well regulated. These plasmids have therefore been useful for a number of different purposes e.g. sections 4.4, 4.5.

4.2 Expression of PRP2 Protein in E. coli

As an alternative to yeast, PRP2 protein was expressed in E. coli. The plasmid expression vector used was pDR540 (Pharmacia), which contains the tac promoter, a fusion of the E. coli trp and lacZ promoters. This promoter responds to synthetic lactose analogues such as IPTG in the same way as the lacZ promoter. The 3kb BamHI fragment from pBM-PRP2, which contains the PRP2 gene was sub-cloned into the BamHI site, downstream of the tac promoter in pDR540, creating pDR-PRP2 (figure 4.5).

The regulation of expression of PRP2 protein in E. coli cells carrying pDR-PRP2 was examined using a similar approach to that used with the yeast expression vectors. E. coli cells carrying pDR-PRP2 were grown to mid-log phase, and expression from the tac promoter was induced by addition of IPTG to the culture (section 2.6.6). After a further brief period of growth cells were harvested, resuspended in PBS, 1mM PMSF, and lysed by sonication. Cellular debris was pelleted by centrifugation, and the supernate and pellet fractions subjected to SDS-PAGE followed by Western blotting. The blot was probed with anti-FP2.3 serum, with the results shown in figure 4.6. When no IPTG was added to the cells a number of strong bands were detected, but no band at approximately 100kD was visible. The strong bands at approximately 80kD and 60kD are possibly due to reaction of the serum with E. coli proteins, which might have been present as contaminants in the antigen preparations which were injected into rabbits. An alternative explanation is that they are proteins which cross-react with rabbit antibodies.

When expression from the tac promoter was induced with IPTG, a band at approximately 100kD was detected in both supernatant and pellet fractions (lanes 2 and 6). When the soluble protein extract was subjected to immunoprecipitation with serum 54 (αFP2.3), using low stringency washing conditions, this band was immunoprecipitated (lane 7). The protein co-migrates with authentic PRP2 protein from yeast extract. No such band is visible in extracts from IPTG-induced cells which carry pDR540 (data not shown). Since the 100kD protein is only present in cells carrying pDR-

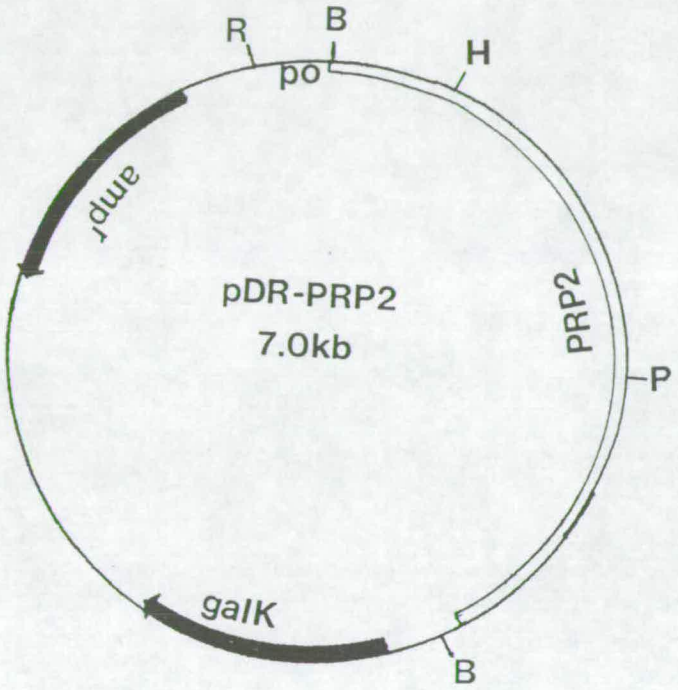


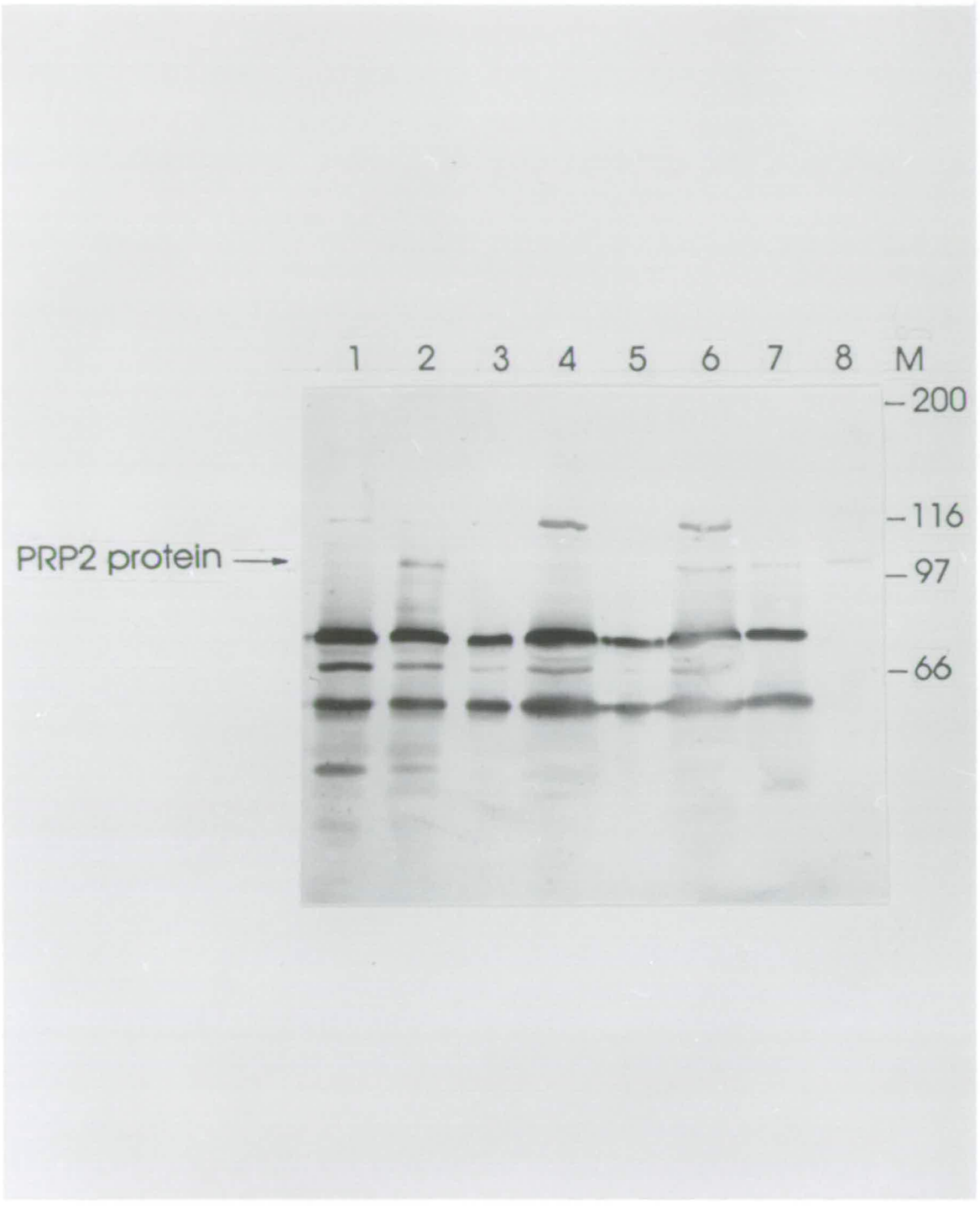
Figure 4.5 Plasmid map of pDR-PRP2

Open boxes: PRP2 coding sequences. Closed boxes: vector genes. Restriction sites: R-EcoRI, B-BamHI, H-HindIII, P-PvuII. p-tac promoter, o-tac operator.

Figure 4.6 Expression of PRP2 protein in E. coli

E.coli cells (strain BMH71-18)carrying pDR-PRP2 were induced for expression of PRP2 protein by addition of IPTG, and were lysed by sonication. Insoluble cell debris was separated by centrifugation. Extracts were analysed by electrophoresis on an 8.5% SDS-polyacrylamide gel followed by Westerm blotting. The blot was probed with rabbit 55 serum (αFP2.3) and developed by the alkaline phosphatase procedure.

Lanes 1, 3 and 5: extracts from cells prior to induction with IPTG. Lanes 2, 4 and 6: extracts from cells after IPTG induction. Lanes 1 and 2: 18μl of resuspended insoluble cell debris. Lanes 3 and 5: 2μl of soluble cell extract. Lanes 4 and 6: 18μl of soluble cell extract. Lane 7: immunoprecipitate from 300μl of soluble cell extract, from IPTG-induced cells, using 5μl of serum 55 (αFP2.3). Lane 8: 1μl of extract from yeast cells carrying pBM-PRP2. Lane M: marker proteins, with sizes indicated in kD.


1 2 3 4 5 6 7 8 M
200
116
97
66
PRP2 protein

PRP2, under inducing conditions, it may be concluded that the 100kD protein in lanes 2, 6 and 7 is PRP2 protein.

It is difficult to compare accurately the relative efficiencies of PRP2 expression in E. coli and yeast, due to the different methods of extract preparation used. Nevertheless, from the concentrations of protein in the extracts in lanes 6 and 8, it is clear that expression of PRP2 protein in E. coli, in this experiment, is at least five times less efficient than in pBM-PRP2-carrying yeast cells.

In many experiments, it has been difficult to detect any expression of PRP2 protein in E. coli, and in only one case was it possible to demonstrate PRP2 protein activity (see section 4.4) in E.coli extracts. One possible cause of this problem is that the initiation of translation of PRP2 mRNA may be inefficient. There is, however, a sequence which is a good match to the Shine and Dalgarno consensus sequence, close to the initiation codon of the PRP2 gene in this construct. The cause of the problem is most likely to be rapid degradation of PRP2 protein by proteases which recognise and degrade foreign proteins. This explanation is supported by the relatively low amounts of FP2.4 and FP2.6 produced in E. coli in comparison to the other, smaller, PRP2-β-galactosidase fusion proteins (section 3.1). Various protease deficient mutant strains of E. coli have been tested for ability to accumulate PRP2 protein, with no significant improvement over the strain used in this experiment (not shown). It is probable that PRP2 protein is extremely rapidly degraded by as yet unidentified E. coli proteases. Because of the unreliable nature of PRP2 expression in E. coli, this system has not been used as a source of PRP2 protein for purification experiments.

4.3 Expression of PRP2 Protein In Vitro

The purpose of expression of PRP2 protein in vitro was to determine whether it interacts directly with spliceosomes, using an experimental design similar to that used by Chang et al (1988). This involves addition of ^{35}S-labelled PRP2 protein to an in vitro splicing reaction, followed by

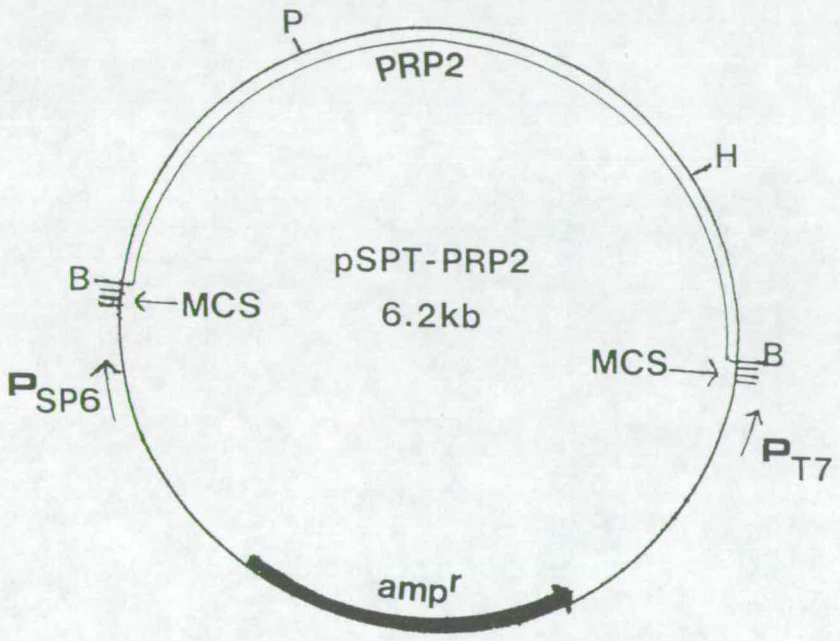


Figure 4.7 Plasmid map of pSPT-PRP2

Open boxes: PRP2 coding sequences. Closed boxes: vector genes. Restriction sites: P-PvuII, B-BamHI, H-HindIII, P_{SP6}-SP6 promoter, P_{T7}-T7 promoter, MCS-multiple cloning site.

non-denaturing electrophoresis to determine whether ^{35}S-labelled PRP2 protein co-migrates with spliceosomes.

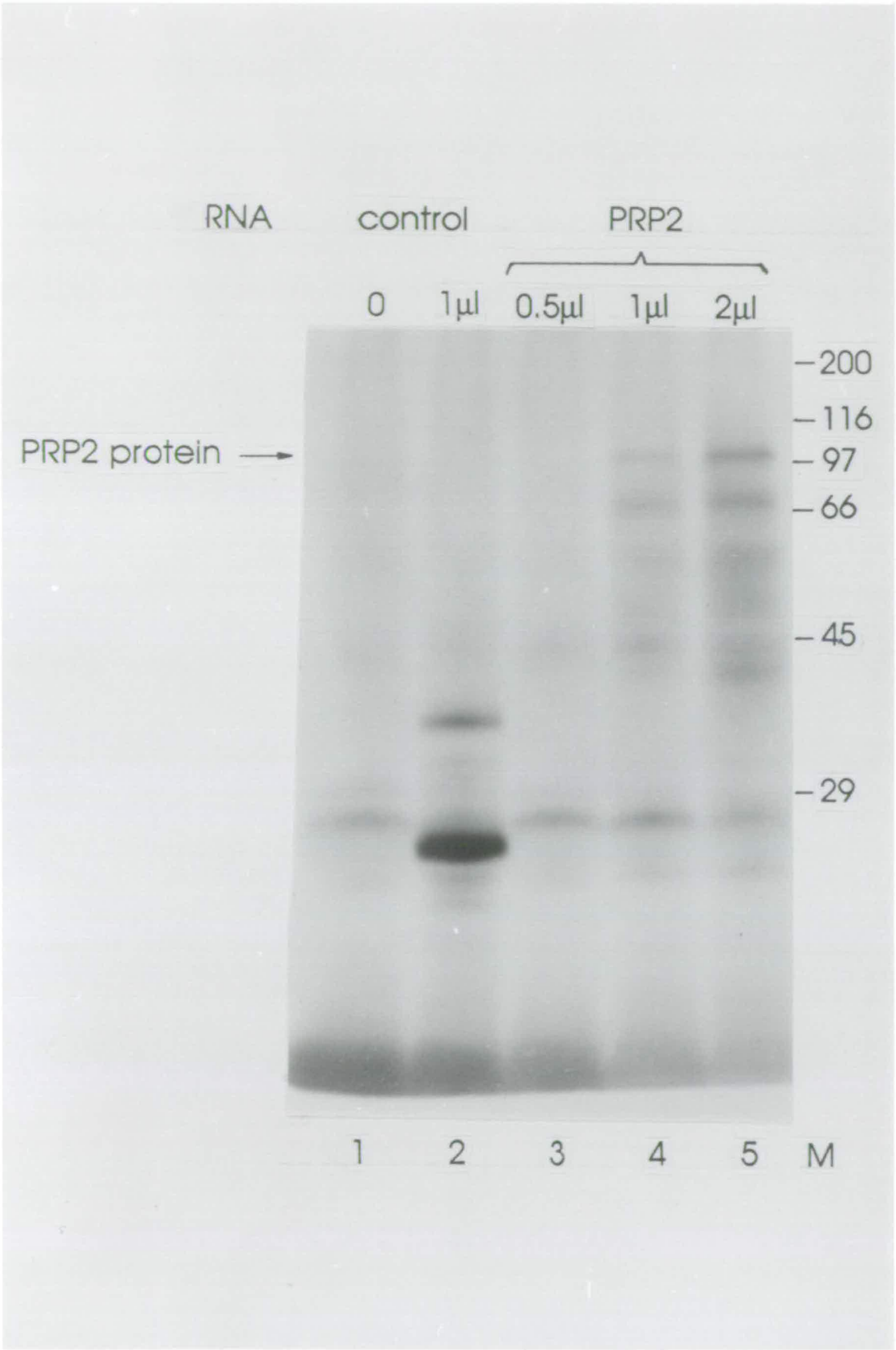
In order to express PRP2 protein by *in vitro* translation, a plasmid designed for transcription of the *PRP2* gene *in vitro* was constructed. This plasmid, pSPT-PRP2 (figure 4.7), contains the whole of the *PRP2* gene inserted downstream of the bacteriophage T7 promoter. It was constructed by inserting the 3kb *Bam*HI fragment from pBM-PRP2 (section 4.1.2), which contains the whole of the *PRP2* gene, into the *Bam*HI site in the polylinker of pSPT18.

pSPT-PRP2 was linearized by digestion with *Sal*I and was transcribed *in vitro* (as described in section 2.4.23, with the exception that the UTP concentration in the transcription reaction was 0.5mM, and no ^{32}P-labelled UTP was present). The RNA made was examined by agarose gel electrophoresis and by denaturing polyacrylamide gel electrophoresis, and was mainly full length (3kb; data not shown). Different sized aliquots of the *in vitro* transcription reaction were used to programme an *in vitro* translation reaction (section 2.6.15), which contained ^{35}S-methionine, and the products of the translation reaction were examined by SDS-PAGE, followed by autoradiography (figure 4.8).

In the absence of added RNA, there are no protein bands in the 100kD region of the gel (lane 1). There is, however, a band at approximately 27kD and a large smear at the bottom of the gel which is haemoglobin, produced from residual haemoglobin mRNA in the rabbit reticulocyte extract. Lane 2 is a reaction to which 1 µl of a control RNA substrate (see section 2.1.6.3) was added. Large amounts of a protein of the expected molecular weight, 26kD, were made, indicating that the rabbit reticulocyte extract was capable of accurately translating exogenous RNA. When progressively larger aliquots of the pSPT-PRP2-programmed *in vitro* transcription reaction were added to the *in vitro* translation extract (lanes 3 to 5), increasing amounts of a 100kD protein were made, plus various smaller proteins. The 100kD protein was efficiently immunoprecipitated by anti-PRP2 antibodies, confirming that it was PRP2 protein (data not shown). The origin of the smaller proteins is unclear, although they are derived from the *PRP2* gene

Figure 4.8 *In vitro* translation of PRP2 protein.

In vitro translations were performed as described in section 2.6.15, and the proteins produced analysed by electrophoresis on an 8.5% SDS-polyacrylamide gel. Following electrophoresis, the gel was fluorographed. Each sample is 8μl of an *in vitro* translation reaction, programmed with the RNA indicated at the top of the lane. Lane M: marker proteins, with sizes indicated in kD.


RNA
control
PRP2
0
1µl
0.5µl
1µl
2µl
PRP2 protein
200
116
97
66
45
29
1
2
3
4
5
M

since they are also precipitated by anti-PRP2 antibodies, albeit with a lesser efficiency than the full sized protein (data not shown). These proteins might arise due to (a) prematurely terminated substrate RNA or (b) premature termination of translation. Of these alternatives, (b) appears to be the most likely since the RNA used as substrate was mainly full length (not shown). While in some experiments the ratio of full sized PRP2 protein to smaller fragments was higher than in the experiment shown, it was not possible to increase this proportion reproducibly by altering *in vitro* translation conditions (concentrations of Mg^{2+} and K^{+}, presence of yeast tRNA) which might affect the frequency of premature translation termination.

A preliminary step towards the objective of determining whether labelled PRP2 protein co-migrates with spliceosomes on non-denaturing gels was to show that the *in vitro* translated protein has PRP2 activity (section 4.4). However, it was not possible to demonstrate any PRP2 activity in *in vitro* translation reactions programmed by PRP2mRNA (not shown). The reason for this is uncertain: it might be due simply to a limitation of the assay system (section 4.4), which is that only small volumes of protein extract (which must therefore be concentrated) can be added to *in vitro* splicing reactions. Attempts to concentrate *in vitro* translated PRP2 protein did not succeed in demonstrating PRP2 activity. An alternative possibility is that the activity of PRP2 protein in splicing may be dependent on post-translational modifications. Because it was not possible to demonstrate that *in vitro* translated PRP2 protein was active in pre-mRNA splicing, the investigation of whether ^{35}S-labelled PRP2 protein is associated with spliceosomes was not pursued.

4.4 Development of an Assay for the Activity of PRP2 Protein

An assay for PRP2 protein activity was developed as part of pilot studies for purification of the protein. The assay system exploits the fact that the activity of splicing extracts made from temperature sensitive *prp2* strains is itself temperature sensitive (Lustig *et al*,1986). It is possible to show that the temperature sensitivity of such extracts is due

Figure 4.9 Assay system for PRP2 protein activity in pre-mRNA splicing.

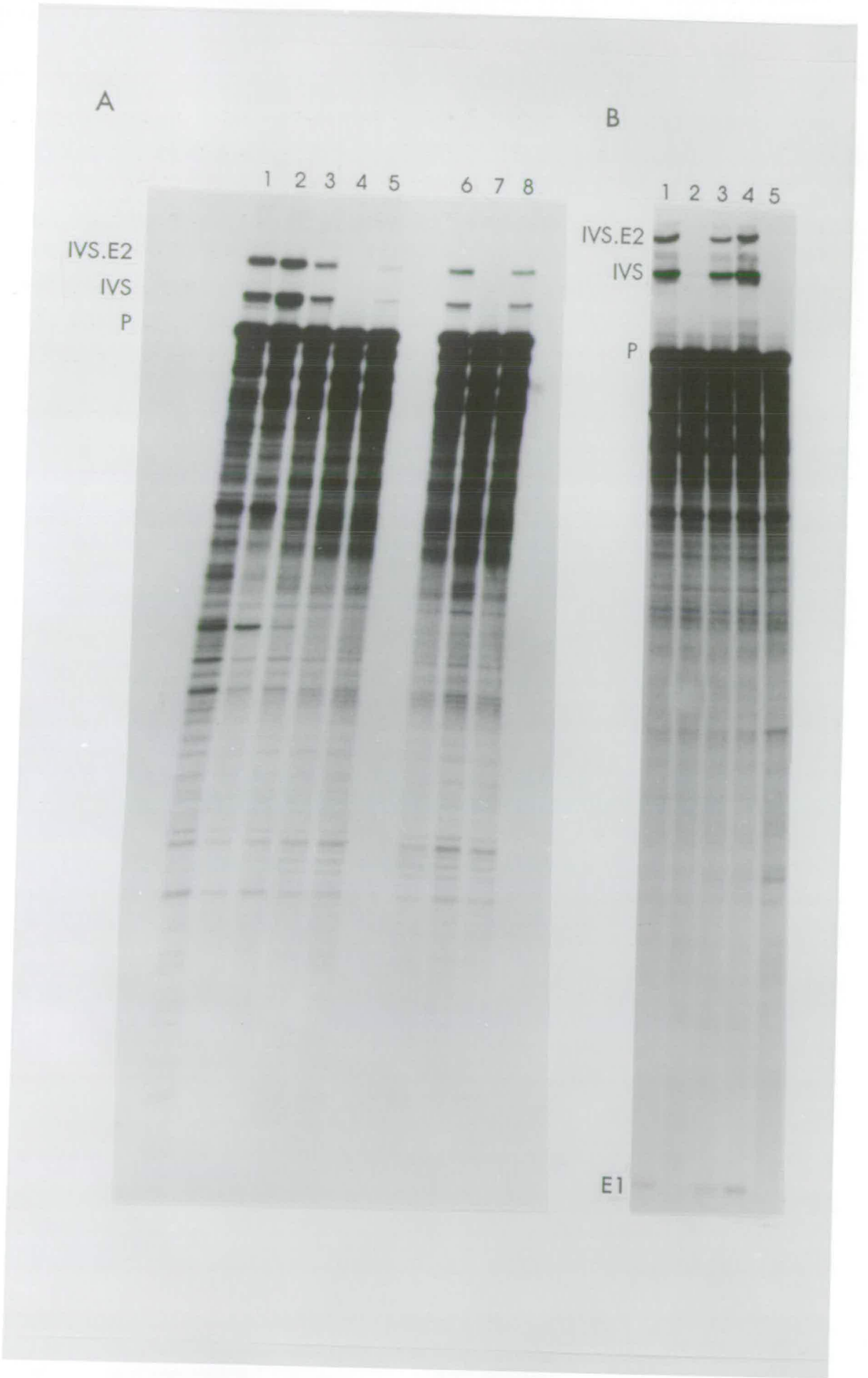
A: Complementation of heat inactivated prp2 splicing extracts with different protein extracts.

Wild type and prp2 splicing extracts (made from strains BJ2412 and JBY27 respectively) were heated for different periods of time at 32°C, prior to *in vitro* splicing. RNA was purified from a 10µl *in vitro* splicing reaction and analysed by electrophoresis on a 12% acrylamide, 8M urea gel. The pre-mRNA used in this experiment was made by *in vitro* transcription of pSPRP51AΔ2, cut with DdeI.

Splicing extracts were heated for 0 (lanes 1 and 3) or 60 (lanes 2 and 4 to 8) minutes at 32°C, prior to commencement of the splicing reaction. Lanes 1 and 2: splicing reactions performed with extract made from wild type (BJ2412) cells. Lanes 3 to 8: splicing reactions performed with extract from *prp2* (JBY27) cells. Following heating, but prior to commencement of the reaction, 1µl of the following was added: dH_2O (lanes 2 and 4); a 1:5 dilution of extract from cells carrying pBM125 (lane 5); a 1:5 dilution of extract from cells carrying pBM-PRP2 (lane 6); a 1:15 dilution of extract from cells carrying pBM125 (lane 7); a 1:15 dilution of extract from cells carrying pBM-PRP2 (lane 8). P-pre-mRNA, IVS.E2-lariat intermediate, IVS-lariat product. Exon 1 and spliced mRNA are not visible in this gel due to the background of degraded pre-mRNA.

B. Splicing activity of complementing extract from cells carrying pBM-PRP2.

Prp2-4 splicing extract (made from strain DJY39) was heated for different periods of time at 32°C, prior to *in vitro* splicing. RNA was purified from a 10µl *in vitro* splicing reaction and analysed by elctrophoresis on a 6% acrylamide, 8M urea gel. The pre-mRNA used in this experiment was made by *in vitro* transcription of pSPRP51A, cut with BamHI.


A
1 2 3 4 5 6 7 8
IVS.E2
IVS
P
B
1 2 3 4 5
IVS.E2
IVS
P
E1

to the temperature sensitive prp2 protein (see below) and that splicing activity can be restored by addition to the heated extracts of wild type PRP2 protein. (This is referred to as in vitro complementation). Such heat inactivated extracts therefore provide an assay system for the activity of PRP2 protein.

An experiment demonstrating that heat inactivation of prp2 extracts was due to the heat inactivation of prp2 protein is shown in figure 4.9A. Wild type and prp2 extracts were incubated at 32°C for 1 hour (section 2.5.3) and small amounts of complementing extracts were added, prior to the commencement of in vitro splicing. Lanes 1 and 2 show that the activity (as measured by the production of lariat containing RNAs) of splicing extract made from a wild type strain of yeast (BJ2412) was not temperature sensitive. The activity of prp2-1 splicing extract was destroyed by a one hour incubation at 32°C (lanes 3 and 4) but was restored by adding 1µl of a 1:5 dilution of extract from cells carrying pBM-PRP2 (lane 6).

(The sources of PRP2 protein used in this experiment were extracts, made by the procedure for preparation of splicing extracts, from KY117 cells carrying either pBM125 or pBM-PRP2, and grown on raffinose plus galactose as carbon sources. These extracts were used in all experiments involving complementation of heat inactivated prp2 splicing extract).

Addition of an equal amount of extract from cells carrying pBM125 complemented the heat inactivated prp2 splicing extract only weakly (lane 5). At a 1:15 dilution the pBM-PRP2 extract was still able to complement the prp2 extract (lane 8), while pBM125 extract did not (lane 7). The fact that an extract which contained elevated levels of PRP2 protein (section 4.1.2) was able to complement the heat inactivated prp2 splicing extract at higher dilutions than control extract, is strong evidence that the temperature sensitivity of the splicing extract is due to the temperature sensitive prp2 protein. In some experiments, however, a process of non-specific heat inactivation of prp2 extracts occurred, and it was therefore necessary to determine whether non-specific heat inactivation occurred in each such experiment, by demonstrating full restoration of activity by small amounts of extracts from cells carrying pBM-PRP2.

Figure 4.9B demonstrates that the complementing extract did not itself possess any splicing activity. The temperature sensitive prp2 splicing extract used in this experiment is made from a prp2-4 strain of yeast. Splicing extracts made from strains carrying this prp2 allele have very similar properties to prp2-1 extracts. Prp2.4 extracts were incubated at 32°C for 1 hour, and small amounts of complementing extracts were added prior to in vitro splicing. As lanes 1 to 3 show, the activity of the prp2-4 splicing extract was temperature sensitive, and could be restored by addition of 1µl of a 1:10 dilution of extract from cells carrying pBM-PRP2. Lane 5 shows that 1µl of pBM-PRP2 extract has no splicing activity in the absence of prp2.4 splicing extract. The restoration of activity to heat inactivated prp2 splicing extracts by pBM-PRP2 extract must therefore be due to PRP2 protein.

In the experiment shown in figure 4.9B, lane 4, the complementing extract was added to the heat inactivated splicing extract after the commencement of the splicing reaction i.e. after the early stages of splicing complex formation have occurred (Lin et al, 1987; Cheng and Abelson, 1987). Since the PRP2 protein was able to complement the prp2 extract under these conditions, it is clear that PRP2 protein does not need to be present at the initial stages of splicing complex formation, in order to perform its function, in agreement with the findings of Cheng and Abelson (1987).

4.5 Partial Purification of PRP2 Protein

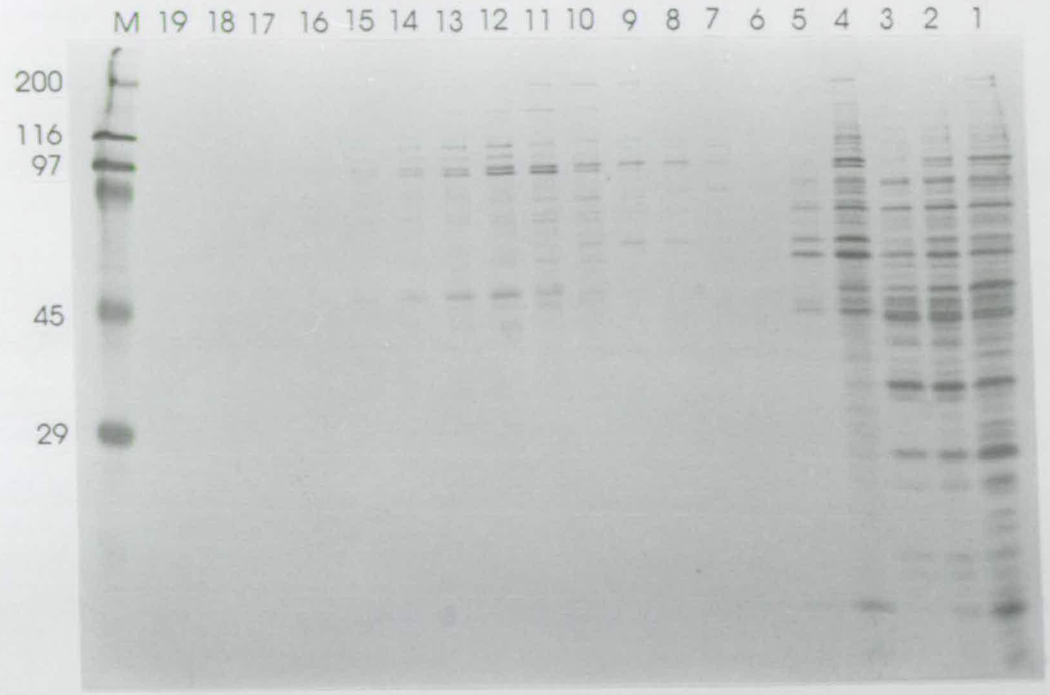
A pilot study for the purification of PRP2 protein is shown in figure 4.10. A 1L culture of DJY40 cells, carrying pKV-PRP2, was grown on yeast minimal medium with raffinose plus galactose as carbon source, in order to induce expression of PRP2 protein (section 4.1.2). The cells were harvested by centrifugation (5000rpm, for 10 minutes in a Sorvall GS-3 rotor), and their cell walls partially digested with lyticase in order to weaken them. They were then lysed by sonication (section 2.6.5). This method of lysis was chosen in order to maximize extraction of PRP2 protein from the cells while minimizing the extraction of other proteins. Partial spheroplasting

Figure 4.10 Partial purification of PRP2 protein.

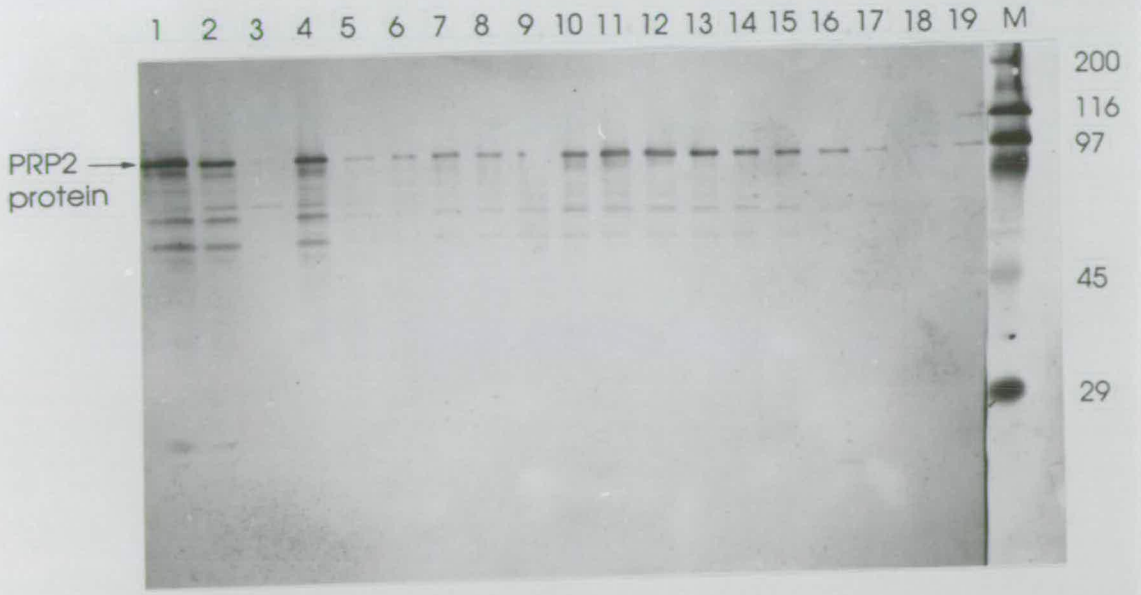
Samples of different fractions from the partial purifgication of PRP2 protein were analysed by electrophoresis on 2 15% SDS-polyacrylamide gels. The gels were stained with Coomassie Blue (A), or western blotted, probed with serum 139 (αFP2.5) and developed by the alkaline phosphatase procedure (B). The quantities given are for A: in B the quantities were 50% of those in A.

Lane 1: unfractionated extract of DJY40 cells carrying pKV-PRP2, 1% of total. Lane 2: supernatant of 38,000rpm centrifugation, 1% of total. Lane 3: supernatant of 50% ammonium sulphate precipitation, 1% of total. Lane 4: pellet of 50% ammonium sulphate precipitation, 1% of total. Lane 5: flowthrough fraction from heparin agarose column, 1% of total. Lanes 6 to 19: heparin agarose column fractions, 5% of total. Lane 6; fraction 2. Lane 7: fraction 4. Lane 8: fraction 6. Lane 9: fraction 8. Lane 10: fraction 10. Lane 11: fraction 12. Lane 12: fraction 14. Lane 13: fraction 16 Lane 14: fraction 18. Lane 15: fraction 20. Lane 16: fraction 22. Lane 17: fraction 24. Lane 18: fraction 26. Lane 19: fraction 28. Lane M: marker proteins, with sizes indicated in kD.

A


M 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
200
116
97
45
29

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M
PRP2
protein
200
116
97
45
29

of the cells was found to increase the yield of protein upon sonication (not shown). However, breaking the cells with a Dounce homogenizer, while further increasing the total protein yield did not increase the yield of PRP2 protein.

Cell debris was removed by centrifugation at 17,000rpm for 30 minutes at 4°C and the extract subjected to a further centrifugation at 38,000rpm for 1 hour at 4°C, in order to remove fragments of chromatin and ribosomes. The extract was analysed by SDS-PAGE and contained proteins in the range 15kD to greater than 200kD (figure 4.10A, lane 1). Some low molecular weight proteins, which were possibly ribosomal proteins or chromatin-associated proteins, were removed by high speed centrifugation (lane 2). PRP2 protein remained in the supernatant after centrifugation (figure 4.10B lane 2).

Ammonium sulphate was then added to the high speed supernate, to 50% saturation, and the protein precipitate recovered by centrifugation (section 2.5.2). Both pellet and precipitate were dialysed against buffer A (20mM HEPES pH 7.9, 0.5mM EDTA, 0.5mM DTT, 10% (v/v) glycerol), to remove ammonium sulphate. As can be seen (figure 4.10A, lanes 3 and 4), this step selectively removes a number of proteins, particularly low molecular weight proteins. PRP2 protein is precipitated essentially quantitatively in this step (figure 4.10B, lanes 3 and 4).

The ammonium sulphate precipitate was loaded onto a 1ml heparin-agarose column, which was pre-equilibrated with buffer A. The flow through fraction from the column was collected and the column washed with 5 column-volumes of buffer A. Proteins were eluted with a 50-800mM sodium chloride gradient (3ml) in buffer A, and 100µl fractions collected. Samples of some of these fractions were analysed by SDS-PAGE (figure 4.10A, lanes 5-19). Although much of the protein in the ammonium sulphate precipitate bound to the heparin agarose, a number of prominent protein bands were found in the heparin agarose flow-through fraction (figure 4.10A, lane 5). PRP2 protein bound to the column, and only a small proportion was found in the flow through fraction (figure 4.10B, lane 5). Elution of proteins from the column with a salt gradient fractionated the

proteins efficiently (figure 4.10A, lanes 6-19). The PRP2 protein peak was found in fractions 10 to 16 which corresponds to a salt concentration range of approximately 300-480 mM.

There is a prominent doublet of protein bands in the 97-100kD range in the crude extract and ammonium sulphate precipitate which clearly fractionate differently on the heparin agarose column, the higher molecular weight protein eluting at lower salt concentrations. The upper band of the doublet is one of the major proteins in the crude extract. It migrates in this gel at 97kD, which is faster than its normal migration rate of approximately 102kD, while PRP2 protein migrates faster than the 97kD marker (figure 4.10B). Such small differences in electrophoretic mobility have been observed for PRP2 protein, in other experiments (not shown). The lower band of the doublet is not generally detected in crude yeast extracts. However, such a band was visible in extracts of pKV-PRP2-carrying cells, but only when such cells had been grown on raffinose plus galactose as carbon source (not shown). This suggests that the lower band of the doublet is PRP2 protein. The suggestion is apparently supported by the coincidence of position of the peak of this protein with the peak of PRP2 protein. Although the intensity of the bands in lane 10 to 16 in figures 4.10A and B is not precisely parallel, in both cases there is a sharp difference in intensity of the bands between lanes 9 and 10, at the start of the peaks, and between lanes 15 and 16, at the end of the peaks. If it is the case that the lower band of the doublet is the PRP2 protein, then it is clear that substantial purification has been achieved (cf lanes 1 and 11): in lanes 11 and 12 this band is the strongest band in the lane. Fractions 10 and 12 were found to contain PRP2 activity (not shown), but since quantitative assays of PRP2 protein activity were not performed, it is not possible to accurately determine the degree of purification of PRP2 protein.

CHAPTER 5

Interaction of PRP2 Protein with snRNPs and Spliceosomes

5.1 Interaction of PRP2 protein with snRNPs

As mentioned in Chapter 1, two of the PRP gene products, PRP4 protein and PRP8 protein have been shown to be associated with snRNPs (Lossky et al 1987, Banroques and Abelson, 1989, Peterson-Bjorn et al 1989). It was therefore of interest to determine whether PRP2 protein was also associated with yeast snRNPs.

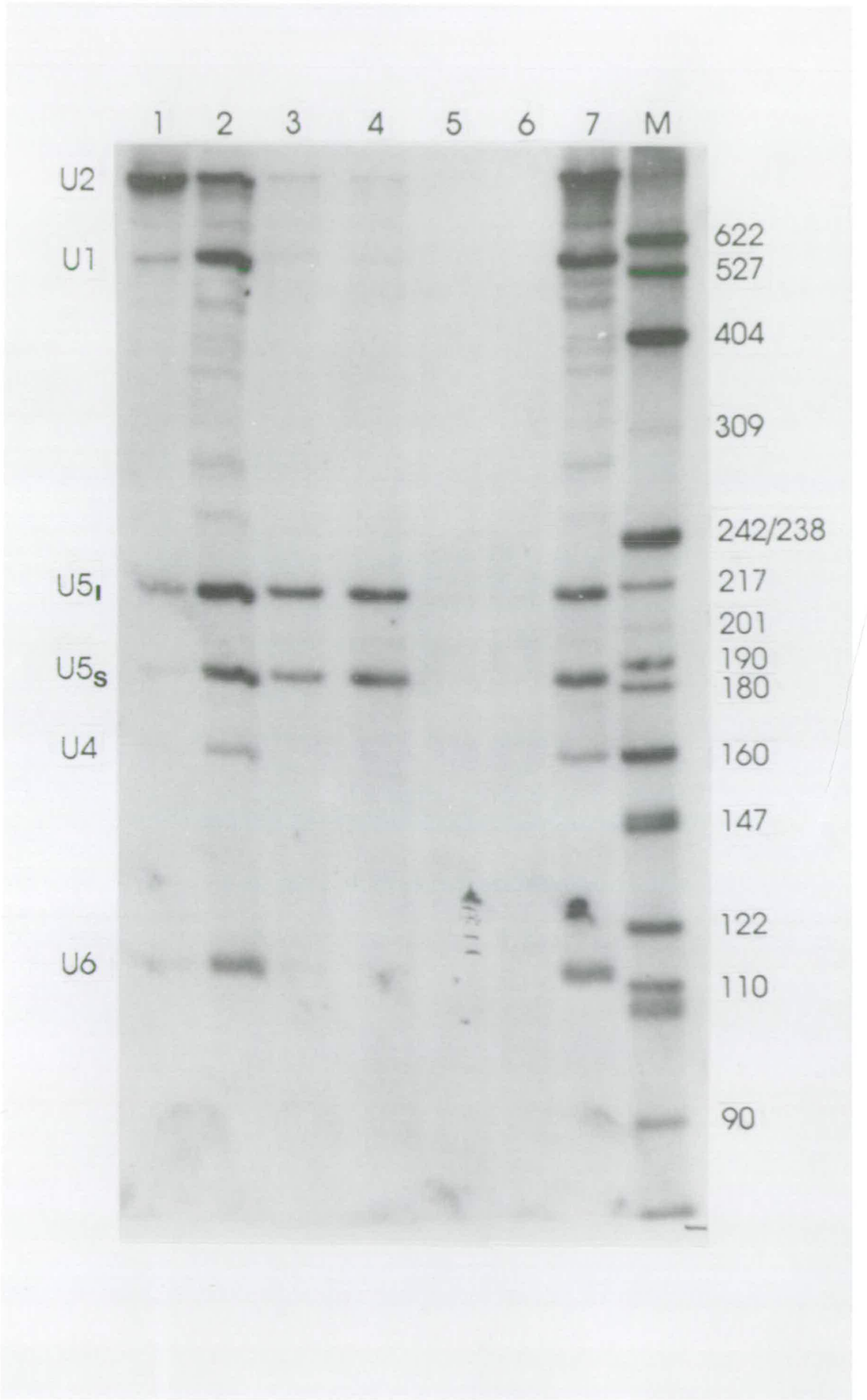
An experimental design similar to that used for PRP4 and PRP8 proteins was employed for this purpose: a yeast snRNP preparation (derived from splicing extract by two consecutive 35% ammonium sulphate precipitations; section 2.5.2) was incubated under 'mock' splicing conditions (identical to conditions for RNA splicing, but with no pre-mRNA added), in the presence or absence of 2mM ATP. Immunoprecipitations, with different antisera, were then performed, using low stringency washing conditions (section 2.6.14.2) and RNA extracted from the immunoprecipitates was analysed by denaturing polyacrylamide gel electrophoresis, followed by Northern blotting (section 2.4.21). The blot was then probed with oligonucleotides complementary to yeast U1, U2, U4, U5 and U6 RNAs, in order to determine if U snRNAs co-precipitated with PRP2 protein.

The results of such an experiment are shown in figure 5.1. Anti-m_3G antibodies immunoprecipitated all 5 U snRNPs from the snRNP preparation (lane 2). Serum 58 (αPRP8) precipitated U5 snRNP, and this did not depend upon whether ATP was present or absent during the incubation of the snRNP preparation, prior to immunoprecipitation (lanes 3 and 4). Affinity purified anti-PRP2 antibodies did not immunoprecipitate any snRNPs, under either conditions (lanes 5 and 6), suggesting that the PRP2 protein is not

Figure 5.1 Immunoprecipitation of snRNPs

A partially purified snRNP preparation was incubated under 'mock splicing' conditions in the presence or absence of ATP, for 20 minutes, and then subjected to immunoprecipitation. RNA was purified from the immunoprecipitates and analysed by electrophoresis on a 6% acrylamide, 8M urea gel. The gel was Northern blotted and the blot probed with ^{32}P-labelled oligonucleotides, complementary to the U snRNPs.

In lanes 3 and 5, no added ATP was present during incubations prior to immunoprecipitation; in lanes 4 and 6, 2mM ATP was present during the incubation. Lane 1: total yeast RNA, 9μg. Immunoprecipitations were performed with anti-m_3G antibodies (3μl; lane 2) serum 58 (3μl; lanes 3 and 4) or anti-PRP2 antibodies (300μl, affinity purified from a mixture of serum 55 and 139; lanes 5 and 6). Lane 7: supernate (equivalent of 10μl 'mock' splicing reaction) from immunoprecipitation in lane 5. Lane M: markers with sizes indicated in nucleotides. The snRNA species U1, 2, 4, 5 and 6 are indicated at the left hand side.


1
2
3
4
5
6
7
M
U2
U1
U5I
U5S
U4
U6
622
527
404
309
242/238
217
201
190
180
160
147
122
110
90

stably associated with an snRNP. Lane 7 demonstrates that all the U snRNPs were present in the supernate from the immunoprecipitation in lane 6, and were not degraded. Since the snRNP preparation used in this experiment does not display an ATP-dependent associatiation of U5 and U4/6 snRNPs (Lossky et al, 1987; Konarska and Sharp, 1987) it appears that it differs from unfractionated splicing extract in some aspects of its response to ATP. The possibility therefore remains that PRP2 protein is associated with snRNPs in the presence of ATP. However, a number of other attempts, using whole splicing extract, to detect association between PRP2 protein and snRNPs, in the presence and absence of ATP, have failed to detect any such association. (In some of these experiments association of U5 snRNP and U4/6 snRNP was detected). A further possibility, that PRP2 protein is very weakly, or transiently associated with snRNPs (other than in spliceosomes), cannot be ruled out.

5.2 Interaction of PRP2 Protein with Spliceosomes

5.2.1. Immunoprecipitation of spliceosomes with anti-PRP2 antibodies

In order to investigate the interaction of PRP2 protein with spliceosomes, a similar approach to that in section 5.1 was used, starting from in vitro splicing reactions, rather than from a snRNP preparation.

An experiment of this type is shown in figure 5.2A. In vitro splicing reactions, using wild type (RP51A) or branchpoint deletion mutant (RP51AΔ3B) pre-mRNAs were incubated for 20 minutes and then subjected to immunoprecipitation (section 2.6.14.2). Lanes 1 to 3 show that intermediates and products of the splicing reaction were formed only when ATP was present and when the pre-mRNA contained a functional UACUAAC sequence. As expected, anti-m_3G antibodies co-precipitate precursor, intermediates and products of the splicing reaction. When these reactions were immunoprecipitated with affinity purified anti-PRP2 antibodies, co-precipitation of significant levels of ^{32}P-labelled RNA species occured only from a standard splicing reaction (lane 7), and not when the pre-mRNA lacks a branchpoint sequence (lane 9), or when ATP is absent from the

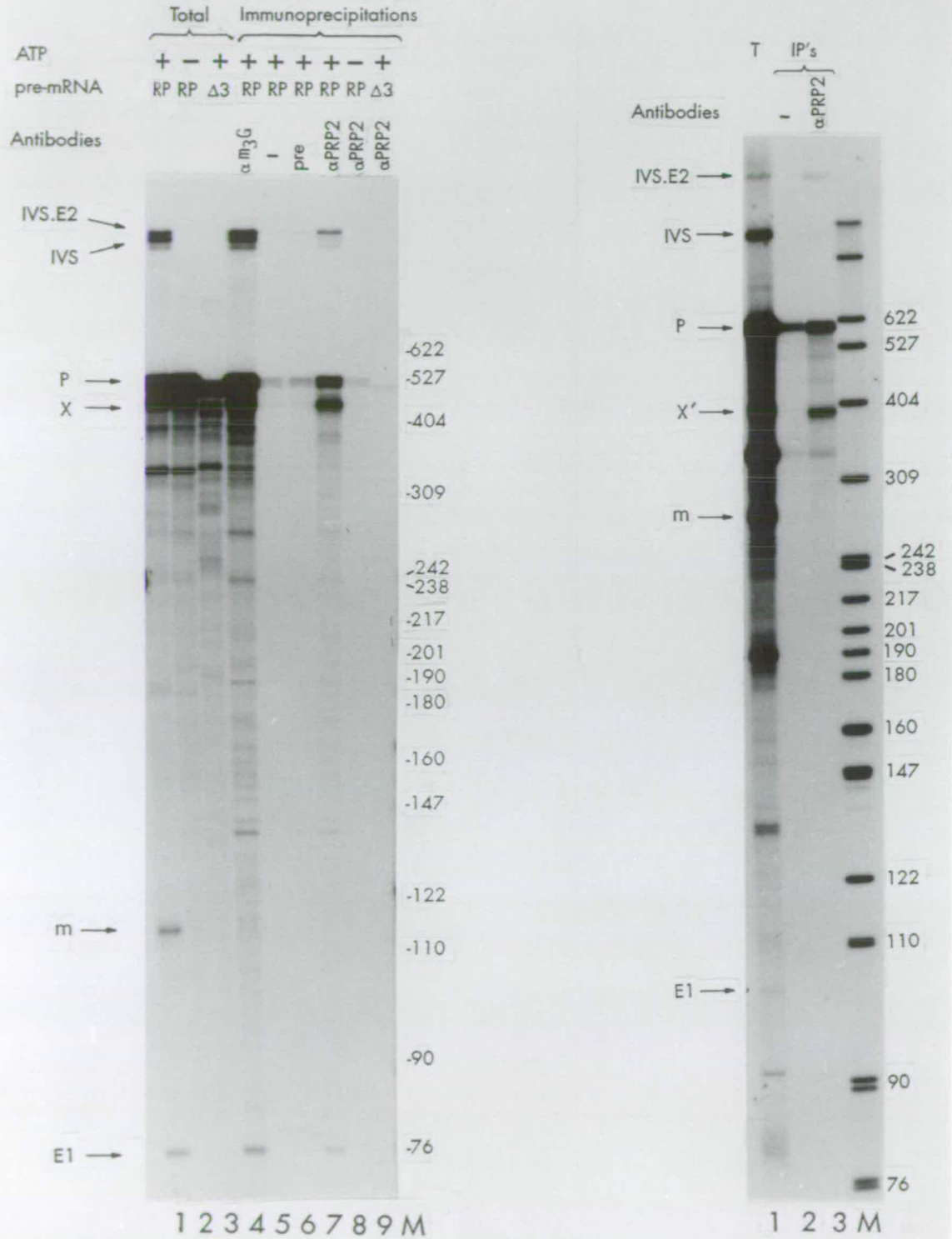
Figure 5.2 Co-precipitation of RNA with PRP2-specific antibodies.

A. Co-precipitation of RP51A RNA. RP51A and RP51A TACTAAC deletion mutant (Δ3B) pre-mRNAs (made by *in vitro* transcription of pSPRP51A and PSPRP51AΔ3B, cut with DdeI) were incubated in *in vitro* splicing reactions (using extract made from BJ2412 cells) for 20 minutes, the reactions stopped and immunoprecipitations performed. RNA was analysed by electrophoresis on a 6% acrylamide, 8M urea gel. The identities of the intermediates and products of the reactions were based on size, ATP dependence and kinetics of appearance (not shown).

Lanes 1 to 3: RNA extracted from 1 μl of unfractionated splicing reactions, performed with RP51A pre-mRNA in the presence (lane 1) or the absence (lane 2) of ATP, or with RP51AΔ3B pre-mRNA (lane 3). Lanes 4 to 9: RNA extracted from immunoprecipitates from 9 μl of splicing reactions; lanes 4 to 7, from reaction 1; lane 8, from reaction 2; lane 9, from reaction 3. Immunoprecipitations were with anti-m_3G antibodies (3μl; lane 4), protein A-Sepharose control (without IgG; lane 5), preimmune IgG (0.5μl; lane 6) or anti-PRP2 antibodies (affinity purified from serum 139, 300μl; lanes 7 to 9). Lane M: markers with sizes indicated in nucleotides. IVS.E2-lariat intermediate, IVS-lariat intron product, P-pre-mRNA, m-mRNA, E1-exon 1.

The upper, stronger pair of bands in the lariat region of the gel are the full size IVS and IVS.E2 species: the lower, fainter band is a degradation product of the IVS.

B. Co-precipitation of actin RNA. Actin pre-mRNA (made by *in vitro* transcription of pSPTact/alu cut with BamHI) was incubated in an *in vitro* splicing reaction for 20 minutes, the reaction stopped and immunoprecipitations performed. RNA was analysed by electrophoresis on a 6% acrylamide, 8M urea gel. Lane 1: RNA extracted from 1 μl of the splicing reaction. Lanes 2,3: RNA extracted from immunoprecipitates from 9μl of the splicing reaction. Immunoprecipitations were with protein A-sepharose alone (lane 2), or anti-PRP2 antibodies (affinity purified from


Total
Immunoprecipitations
ATP
+ − + + + + + − +
pre-mRNA
RP RP Δ3 RP RP RP RP RP Δ3
Antibodies
α m3G
−
pre
αPRP2
αPRP2
αPRP2
IVS.E2
IVS
P
X
m
E1
622
527
404
309
242
238
217
201
190
180
160
147
122
110
90
76
1 2 3 4 5 6 7 8 9 M
T
IP's
Antibodies
−
αPRP2
IVS.E2
IVS
P
X′
m
E1
622
527
404
309
242
238
217
201
190
180
160
147
122
110
90
76
1 2 3 M

splicing reaction (lane 8). This co-precipitation was dependent upon anti-PRP2 antibodies, and did not occur when there were no antibodies (lane 5), or preimmune antibodies (lane 6), bound to the protein A-sepharose. Since spliceosomes form only under standard splicing conditions, it may be concluded that the observed co-precipitation of ^{32}P-labelled RNA in lane 7 is due to co-precipitation of spliceosomes, and not of non-specific RNA-protein complexes. Examination of the pattern of co-precipitated RNA species confirms this conclusion: anti-PRP2 antibodies co-precipitated only precursor and intermediate species, which, by definition, occur only in spliceosomes. A similar pattern of co-precipitation with anti-PRP2 antibodies was observed using an actin pre-mRNA (figure 5.2B, lane 3). In this case, co-precipitation of exon 1 was detected only after a long exposure of the gel (not shown). Further experiments (not shown), demonstrate that anti-PRP2 antibodies purified from rabbit 55 serum (αFP2.3) also precipitate spliceosomes, and specifically co-precipitate the species X with high efficiency (see below).

These observations indicate that PRP2 protein associates with spliceosomes prior to step 1 of the splicing reaction and is present throughout step 1. It is not clear, however, whether PRP2 protein dissociates from the spliceosome before step 2 of the reaction or immediately after completion of this step.

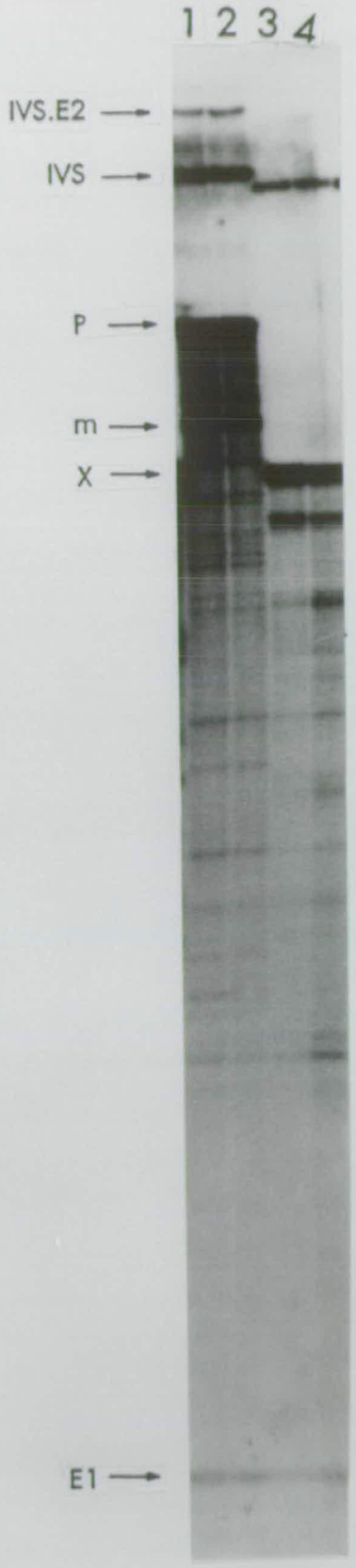
An interesting feature of the pattern of RNA co-precipitation with anti-PRP2 antibodies, is the presence of the species X, at levels approximately tenfold above those expected from its levels in the unfractionated RNA (lane 1). The RNA X is produced during SP6 transcription of the substrate RNA . (In these experiments the full length substrate RNA was not gel purified). By its size, X would be expected to terminate between the UACUAAC sequence and the 3' splice site. If this is true, the results of Rymond and Rosbash (1985) predict that X should undergo step 1, but not step 2, of the splicing reaction

X was gel purified and used as the substrate of a splicing reaction (figure 5.3). Lanes 3 and 4 demonstrate that X undergoes step 1 of the splicing reaction, generating exon 1 and a species which runs slightly

Figure 5.3 *In vitro* splicing of purified species X

Full length RP51A pre-mRNA (made by *in vitro* transcription of pSPRP51A, cut with BamHI), and species X were purified from a denaturing polyacrylamide gel (section 2.4.9) and used as substrates for *in vitro* splicing. RNA was purified from a 10μl *in vitro* splicing reaction and analysed by electrophoresis on a 6% acrylamide, 8M urea gel. IVS.E2-lariat intermediate, IVS-lariat intron product, P-pre-mRNA, m-mRNA, E1-exon 1.

Lanes 1 and 2: duplicate reactions, with full length pre-mRNA as substrate.
Lanes 3 and 4: duplicate reactions, with X as substrate. IVS.E2-lariat intermediate, IVS-lariat intron product, P-pre-mRNA, m-mRNA, E1-exon 1.


1 2 3 4
IVS.E2
IVS
P
m
X
E1

ahead of the full sized lariat product species as would be expected for a lariat RNA with a shortened 3' tail. This experiment therefore confirms that the 3' end of the species X lies between the UACUAAC sequence and the 3' splice site.

In figure 5.2B, lane 3 a similar effect to that in figure 5.2A is observed, with actin pre-mRNA: a species X', the 3' end of which maps between the UACUAAC sequence and the 3' splice site is precipitated at approximately ten times the expected levels. This result suggests that the phenomenon of preferential co-precipitation of RNA species terminating between the UACUAAC sequence and the 3' splice site, is a general one, and is not restricted to RP51A pre-mRNA.

In order to investigate this effect in more detail, two deoxyoligonucleotides (A and C, corresponding exactly to oligonucleotides A and C used by Rymond et al, 1987; figure 5.4), which are complementary to sequences in the region of the RP51A transcript between the UACUAAC sequence and the 3' splice site, were hybridized to the RP51A transcript. The transcripts were then cleaved with E. coli RNase H and used as substrates for in vitro splicing (figure 5.5). The splicing reactions were incubated for 20 minutes, and immunoprecipitations performed with affinity purified anti-PRP2 antibodies.

In agreement with the results of Rymond et al (1987), oligo C-cleaved transcripts (lane 2) efficiently undergo step 1 of the reaction (cf figure 5.3, lanes 3 and 4), while oligo A-cleaved substrates do not (lane 3). The 3' end of X lies between the oligo C cleavage site and that of oligo A, at 32-35 nucleotides downstream of the UACUAAC sequence.

Lanes 4 to 9 show immunoprecipitations performed upon these reactions. Species X (lanes 4 and 7) and oligo A-cleaved substrate (lanes 6 and 9) were preferentially co-precipitated by anti-PRP2 antibodies. Oligo C-cleaved transcripts, in contrast, are co-precipitated with the same efficiency as full length transcripts (lanes 5 and 8). These data show that preferential co-precipitation of pre-mRNAs only occurs when the pre-mRNA contains less than 32-35 downstream from the UACUAAC sequence.

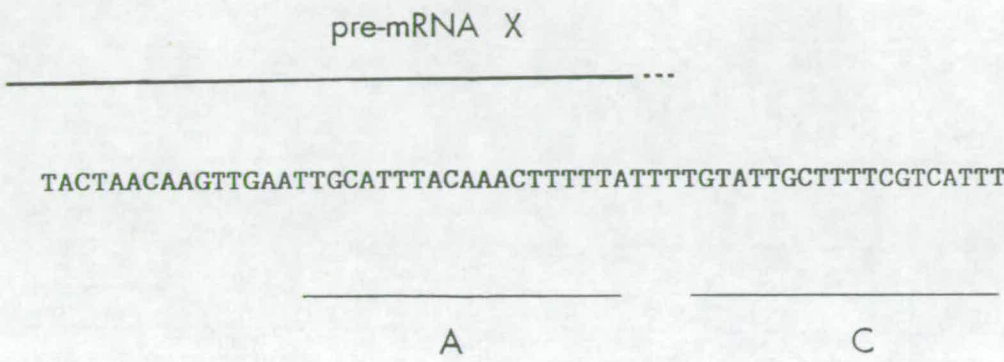


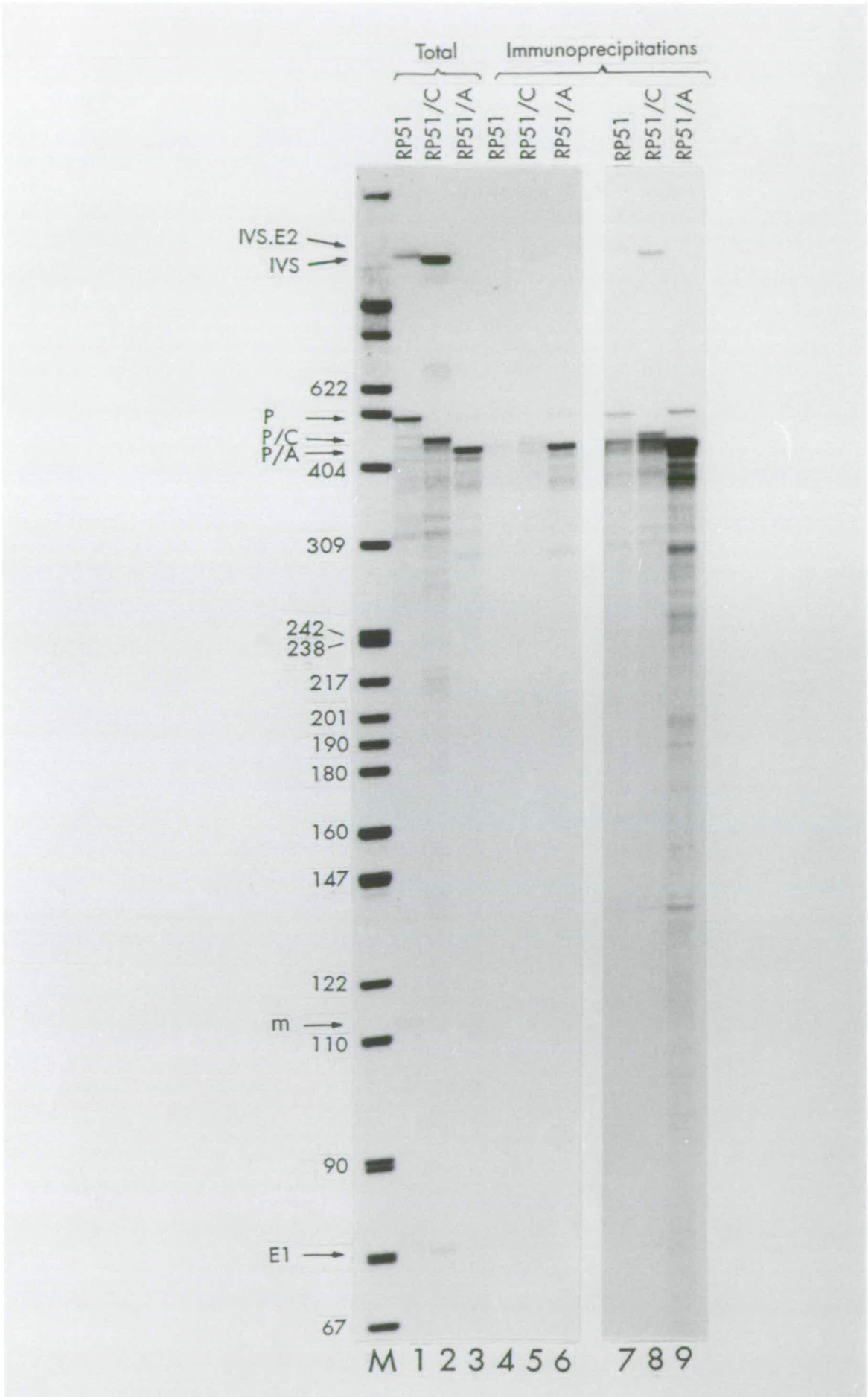
Figure 5.4 Sequence from UACUAAC to 3' splice site of the RP51A gene (from Teem and Rosbash, 1983).

Regions of hybridization of oligonucleotides A and C and approximate position of 3' ends of species X (see figure 5.5) are indicated.

Figure 5.5 Preferential co-precipitation of transcripts terminating between the UACUAAC sequence and the 3' splice site of the RP51A pre-mRNA.

RP51A pre-mRNA (made by *in vitro* transcription of pSPRP51A cut with DdeI) was cleaved with oligonucleotide A or C plus RNase H (section 2.4.22) and used as substrate for *in vitro* splicing. After 20 minutes of incubation, the reactions were stopped and immunoprecipitations performed with anti-PRP2 antibodies (affinity purified from serum 139, 300µl). RNA was analysed by electrophoresis on a 6% acrylamide, 8M urea gel.

Lanes 1 to 3: RNA from 1 µl of unfractionated reactions using full length pre-mRNA (lane 1), oligo C cleaved pre-mRNA (lane 2), or oligo A-cleaved pre-mRNA (lane 3). Lanes 4 to 6: RNA extracted from anti-PRP2 immunoprecipitations, from 9 µl of reactions in lanes 1 to 3, respectively. Lanes 7 to 9: long exposure of lanes 4 to 6. Lane M: markers, with sizes indicated in nucleotides. IVS.E2-lariat intermediate, IVS-lariat intron product, P-pre-mRNA, m-mRNA, E1-exon 1, P/A-oligo A-cleaved pre-mRNA, P/C-oligo C-cleaved pre-mRNA.


Total
Immunoprecipitations
RP51
RP51/C
RP51/A
RP51
RP51/C
RP51/A
RP51
RP51/C
RP51/A
IVS.E2
IVS
622
P
P/C
P/A
404
309
242
238
217
201
190
180
160
147
122
m
110
90
E1
67
M 1 2 3 4 5 6 7 8 9

5.2.2 Heat inactivated prp2 protein is not associated with spliceosomes

The increased sensitivity of detection of co-precipitation available with oligo A-cleaved transcripts was utilized to determine whether heat inactivated prp2 protein associates with spliceosomal complexes. Rymond et al (1987) demonstrated that complexes I and III form on oligo A-cleaved pre-mRNA. Splicing extract made from a prp2-1 strain was inactivated by heating for 1 hour at 32°C (see section 4.4), and used for in vitro splicing reactions with both full length RP51A pre-mRNA, and oligo A-cleaved pre-mRNA. The splicing reactions were incubated for 20 minutes, and immunoprecipitations performed with affinity purified anti-PRP2 antibodies.

Lanes 1 to 3 demonstrate that the extract was efficiently heat inactivated, but was capable of being complemented by a small amount of pBM-PRP2 extract i.e. the heat inactivation was prp2-specific(section 4.4). A similar set of reactions was performed with the same batch of heat inactivated extract, using oligo A-cleaved substrate (lanes 4-6) and immunoprecipitations were performed upon these reactions (lanes 7-10). When prp2-1 protein was heat inactivated (lane 5) no co-precipitation of substrate RNA was observed (lane 9). When the prp2 deficiency was complemented with wild type PRP2 protein, however, (lanes 6 and 10) co-precipitation of substrate RNA was restored.

In a separate experiment (figure 5.7), prp2-1 (lanes 1 and 2) and prp2-4 (lanes 3 and 4) splicing extracts were heat inactivated and subjected to immunoprecipitation with serum 139 (the heat inactivation of the prp2-1 extract is not presented; the heat inactivation of the prp2-4 extract is shown in figure 4.9B). As lanes 2 and 4 demonstrate, heat inactivated prp2 proteins are immunoprecipitated, after heat inactivation, by anti-PRP2 serum. It is therefore clear that the failure to co-precipitate spliceosomes from heat inactivated prp2 extracts, with anti-PRP2 antibodies is not due to failure to immunoprecipitate the heat inactivated prp2 protein.

These results provide further evidence that co-precipitation of substrate RNA is dependent upon PRP2 protein, and demonstrate that heat inactivation

Figure 5.6 Immunoprecipitation of spliceosomal complexes from heat inactivated prp2-1 splicing extract.

RP51A pre-mRNA (made by *in vitro* transcription of pSPRP51A cut with DdeI), or RP51A pre-mRNA cleaved with oligonucleotide A plus RNase H (section 2.4.22) was used as substrate for *in vitro* splicing, with prp2-1 splicing extracts (made from strain DJY85). After 20 minutes of incubation the reactions were stopped and immunoprecipitations performed with anti-PRP2 antibodies (affinity purified from serum 139, 400µl). RNA was analysed by electrophoresis on a 6% acrylamide, 8M urea gel.

Lanes 1 to 6: RNA extracted from 1µl of unfractionated reactions. Lanes 1 to 3: reactions with full length RP51A substrate, performed with untreated prp2-1 extract (lane 1), heat treated prp2-1 extract (lane 2), or heat treated prp2-1 extract complemented with wild type PRP2 protein (section 4.4; lane 3). Lanes 4 to 6 are as lanes 1 to 3, but using oligo A-truncated RP51A pre-mRNA. Lane 7: protein A-Sepharose control immunoprecipitation from 9µl of reaction in lane 4. Lanes 8-10: anti-PRP2 immunoprecipitations from 9µl of reactions in lanes 4-6 respectively. M: markers with sizes indicated in nucleotides. IVS.E2-lariat intermediate, IVS-lariat intron product, P-pre-mRNA, m-mRNA, E1-exon 1, P/A-oligo A-cleaved pre-mRNA.

The origin of the band at approximately 370 nucleotides in lanes 8 and 10 is uncertain: while generally present in such experiments its intensity is variable.

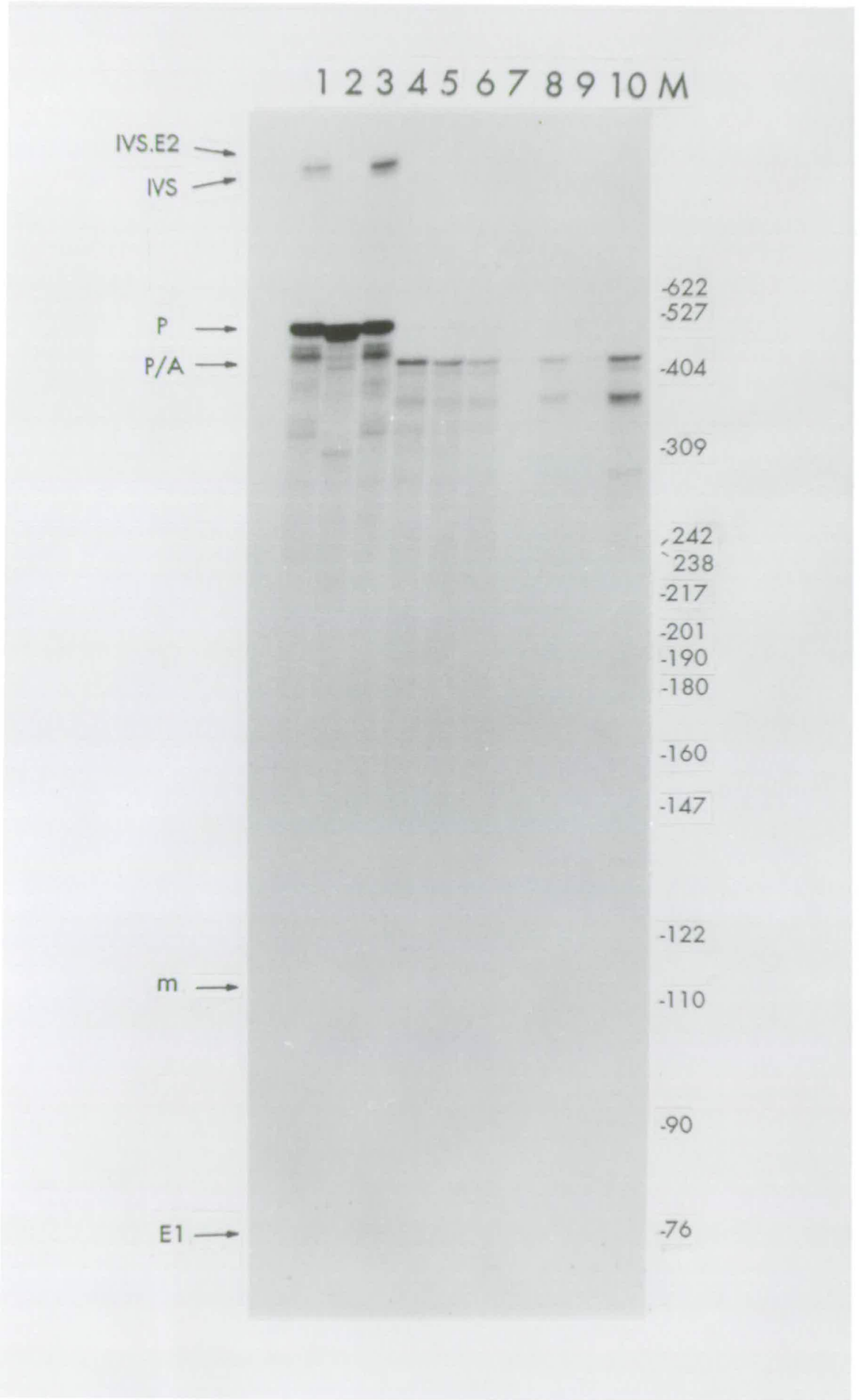
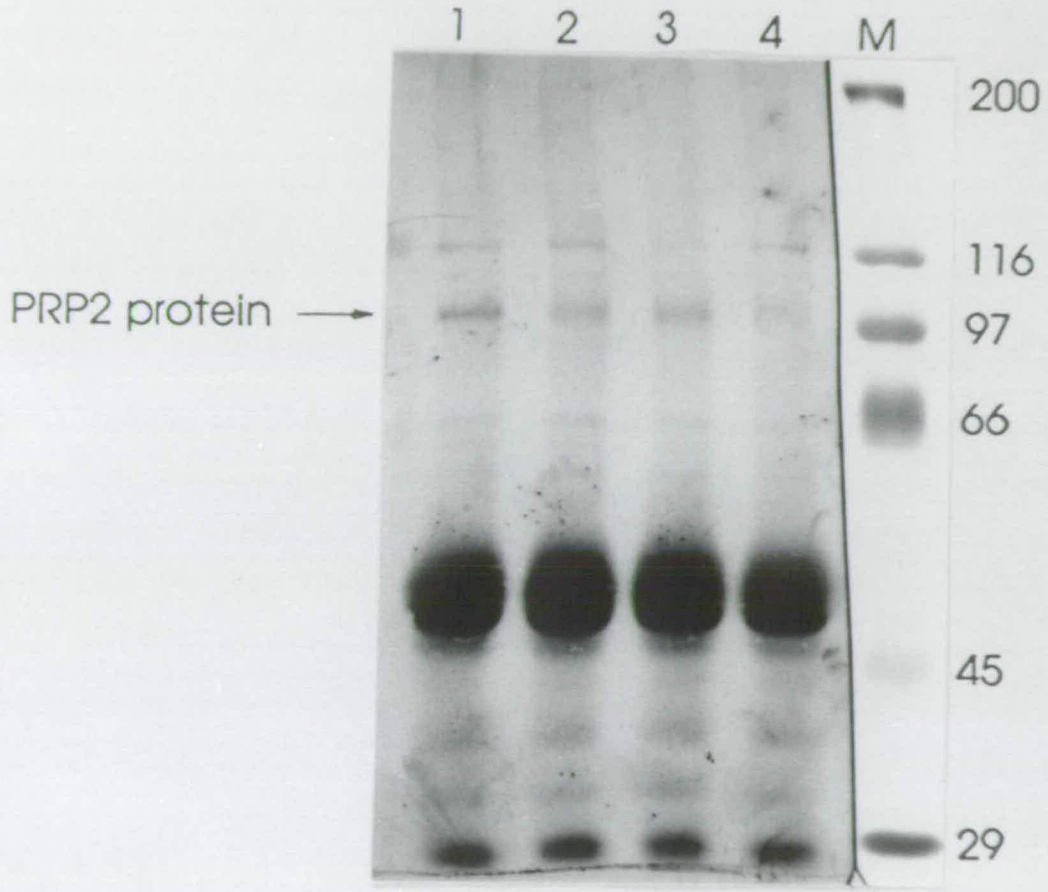

1 2 3 4 5 6 7 8 9 10 M
IVS.E2
IVS
P
P/A
m
E1
-622
-527
-404
-309
242
238
-217
-201
-190
-180
-160
-147
-122
-110
-90
-76

Figure 5.7 Immunoprecipitation of heat-inactivated prp2 proteins.

Splicing extracts from prp2-1 cells (strain JBY27) or prp2-4 cells (strain DJY39) were heat inactivated (section 4.4) and used for *in vitro* splicing. Immunoprecipitations were performed, upon 20µl splicing reactions, using 2µl of serum 139 (αFP2.5) using low stringency washing conditions. The immunoprecipitates were analysed by electrophoresis on an 8.5% SDS-polyacrylamide gel, followed by Western blotting. The blot was probed with rabbit 139 serum (αFP2.5) and developed by the alkaline phosphatase procedure.

Prp2-1 (lanes 1 and 2) or prp2-4 (lanes 3 and 4) splicing extracts were heat treated for 0 (lanes 1 and 3) or 60 (lanes 2 and 4) minutes at 32°C. M: marker proteins, with sizes indicated in kD.


1
2
3
4
M
200
116
97
66
45
29
PRP2 protein

of prp2-1 protein prevents its interaction with spliceosomal complexes. Similar results have been obtained with extracts made from prp2-4 yeast strains (not shown).

5.2.3 The kinetics of interaction of PRP2 protein with spliceosomes

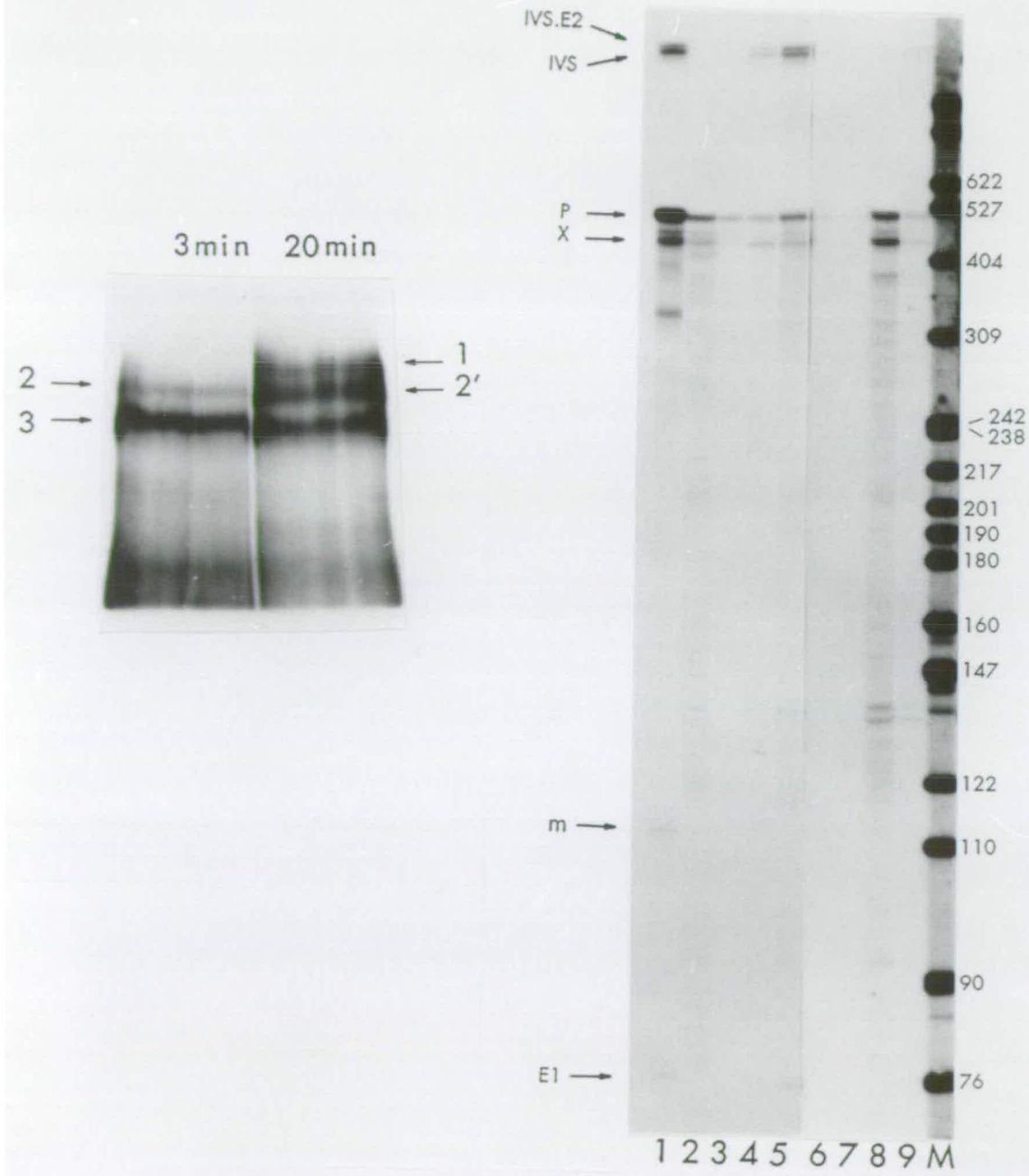
To investigate which specific spliceosomal complexes PRP2 interacts with, different spliceosomal complexes formed on RP51A substrate RNA were fractionated by non-denaturing gel electrophoresis. A typical time course of appearance of the different complexes is shown in figure 5.8A. After three minutes of incubation, two complexes, labelled 2 and 3 are present, while after twenty minutes a further complex, 1 is visible. These complexes were electroeluted (section 2.5.5) and 10% of the ^{32}P-labelled RNA analysed by denaturing gel electrophoresis (figure 5.8B). Complexes 3 and 2 contain only pre-mRNA (lanes 2,3). Complex 1 contains mainly pre-mRNA plus a small amount of intermediates and lariat product, while after 20 minutes complex 2' contains the majority of intermediates, plus some pre-mRNA (lanes 4 and 5). It is possible that in addition to active spliceosomes, this band contains some residual complex 2, since complexes 2 and 2' co-migrate in the non-denaturing gel. The ratio of X to full length pre-mRNA is greatest in complex 1, suggesting that X is trapped in this complex, its progress to complex 2' possibly being retarded due to the lack of a sufficient length of RNA 3' to the UACUAAC sequence (see Chapter 6)

Anti-PRP2 immunoprecipitations were performed on the remaining 90% of the material eluted from the non-denaturing gel, (figure 5.8B, lanes 6-9). While there is no detectable immunoprecipitation from complexes 2 and 3, strong co-precipitation of pre-mRNA is observed from complex 1, and weaker co-precipitation from complex 2'. It is therefore likely that the majority of pre-mRNA and species X co-precipitated from whole splicing reactions comes from complex 1. For reasons which are not clear, co-precipitation of splicing intermediates in elution experiments is relatively inefficient. However, since intermediates are precipitated from whole splicing reactions, PRP2 protein must be associated with this complex. These observations indicate that the point of initial interaction of PRP2 protein with spliceosomal complexes is the transition from complex 2 to complex 1.

Figure 5.8 Kinetics of interaction of PRP2 protein with spliceosomes.

A: kinetics of splicing complex formation. 20µl *in vitro* splicing reactions using RP51A pre-mRNA (made by *in vitro* transcription of pSPRP51A cut with DdeI) were incubated for the times indicated and then quenched on ice and fractionated by non-denaturing gel electrophoresis (section 2.5.4) for 8 hours at 120V.

B: Elution and immunoprecipitation of splicing complexes from a non-denaturing gel. Bands 3, 2, 1 and 2' were eluted from the gel shown in figure 5.8A, and 10% of the eluted RNA was purified (lanes 2 to 5 respectively). The remaining 90% was immunoprecipitated with anti-PRP2 antibodies (affinity purified from serum 139, 300µl; lanes 6 to 9, respectively). RNA was analysed by electrophoresis on a 6% acrylamide, 8M urea gel. Lane 1: RNA extracted from 1 µl of the unfractionated 20 minute sample of the splicing reaction. Lanes 1 to 5 are a 24 hour exposure; lanes 6 to 10 are a 1 week exposure. M: markers, with sizes indicated in nucleotides. IVS.E2-lariat intermediate, IVS-lariat intron product, P-pre-mRNA, m-mRNA, E1-exon 1.


3min
20min
2
3
1
2′
IVS.E2
IVS
P
X
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242
238
217
201
190
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160
147
122
110
90
76
1 2 3 4 5 6 7 8 9 M

5.2.4 Localisation of PRP2 protein in spliceosomes

A method of partially disrupting spliceosomes was used to investigate the localisation of PRP2 protein, relative to pre-mRNA, in spliceosomes. The experimental design (figure 5.9) exploits the fact that the central region of the RP51A intron is available, in spliceosomes, for hybridization with oligonucleotides (Rymond and Rosbash, 1986). Since yeast splicing extracts contain an endogenous RNase H activity, adding to a splicing reaction an oligonucleotide complementary to a central region of the intron results in cleavage of the pre-mRNA into two fragments. The cleaved spliceosomes are then subjected to immunoprecipitation under standard conditions.

An experiment of this type is shown in figure 5.10. *In vitro* splicing reactions were incubated for 15 minutes under standard conditions, and for a further 4 minutes in the presence of oligonucleotide E. As lanes 3 and 4 demonstrate, addition of oligonucleotide E efficiently cleaves the pre-mRNA to give the two major species indicated. These species were identified on the basis of their size, and further cleavage with oligonucleotide C (lane 11). In agreement with the findings of Rymond and Rosbash (1986), after cleavage, the 5' portion of the molecule is present in much larger amounts than the 3' portion, due presumably to degradation of the 3' portion of the molecule by endogenous 5' to 3' exonucleases (Rymond and Rosbash, 1986). The small discrepancy between the sizes of the 5' portions of wild type and Δ3B RNA is due to a minor difference in sequence, introduced during the construction of the Δ3B deletion (Pikielny et al., 1983).

The cleaved spliceosomes were subjected to immunoprecipitation with anti-m_3G or anti-PRP2 antibodies (lanes 6 and 7). Anti m_3G antibodies co-precipitated both the 5' and 3' portions of the pre-mRNA plus residual uncleaved pre-mRNA and exon 1 (lane 6). Despite the degradation of most of the population of 3' portions of pre-mRNA there therefore exists a certain quantity of 3' portions associated with snRNPs. In contrast to the result with anti-m_3G antibodies, anti-PRP2 antibodies co-precipitated only the 5' portion of the pre-mRNA (lane 7), and this precipitation was

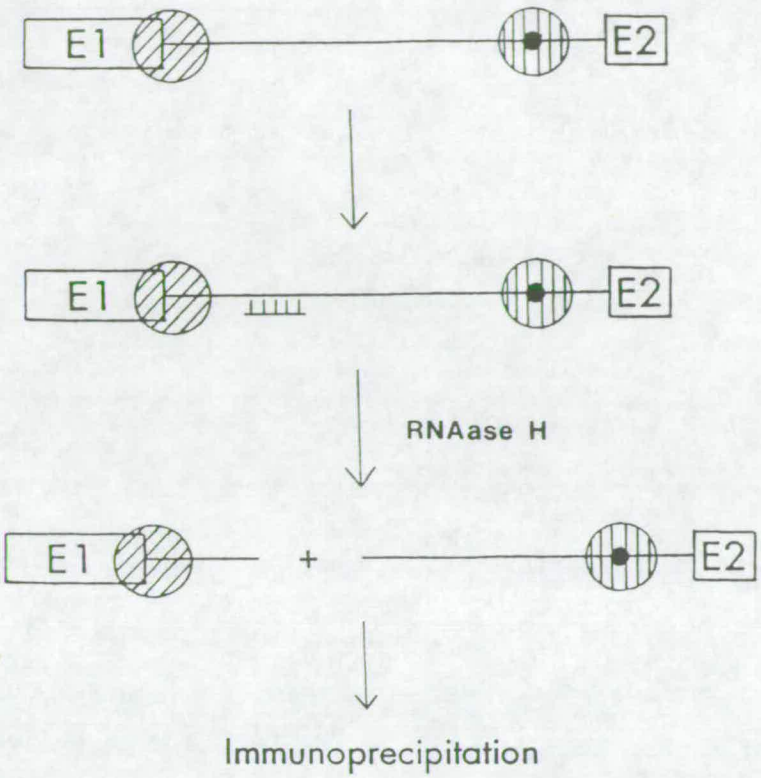


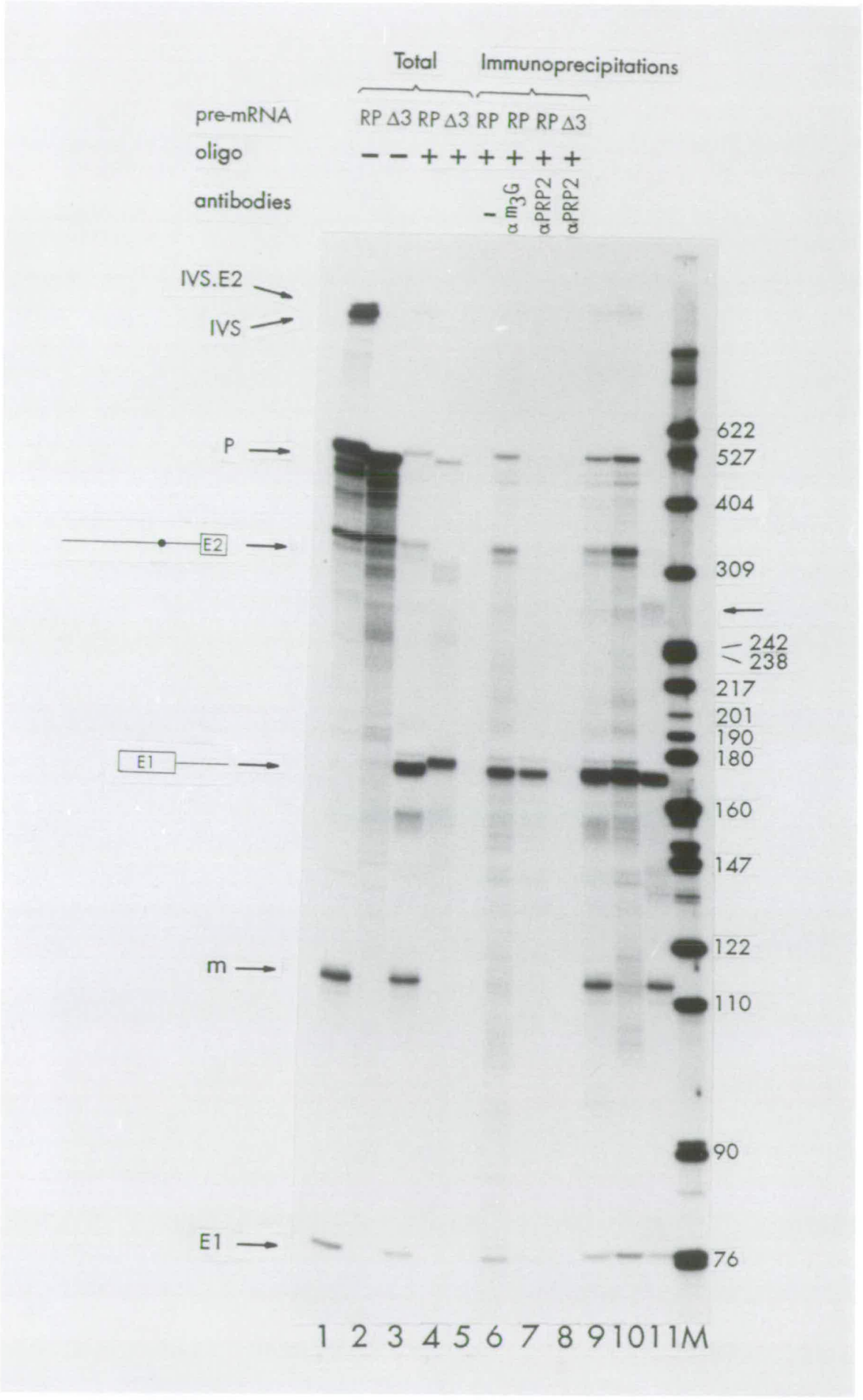
Figure 5.9 Procedure for cleavage of RNA and immunoprecipitation of splicing complexes.

The oligonucleotide used, E, hybridizes to the RP51A intron sequence between 90 and 110 nucleotides downstream of the 5' splice site. The large dot indicates the position of the TACTAAC sequence and the shaded circles indicate complexes which protect these regions of the RNA (Rymond and Rosbash, 1986). Separate complexes are drawn at each site for reasons of clarity.

Figure 5.10 Immunoprecipitation of cleaved spliceosomes

In vitro splicing reactions using RP51A or RP51AΔ3B pre-mRNA (made by *in vitro* transcription of pSPRP51A or pSPRP51AΔ3B cut with DdeI) were incubated for 15 minutes under standard conditions and then for a further 4 minutes with oligonucleotide E (0.2nmol). The reactions were stopped and immunoprecipitations performed, using low stringency washing conditions. RNA was analysed by electrophoresis on a 6% acrylamide, 8M urea gel.

Lane 1: RNA from 1 µl of reaction using full length RP51A pre-mRNA, sample taken prior to addition of oligo E. Lane 2: as lane 1, from reaction using Δ3B pre-mRNA. Lanes 3 and 4: RNA from 1 µl of reactions in lanes 1 and 2, respectively, samples taken after incubation with oligonucleotide E. Lanes 5 to 7: RNA extracted from immunoprecipitates from 9 µl of reaction in lane 3. Immunoprecipitations were with protein A-Sepharose (lane 5), anti-m_3G antibodies (3µl; lane 6), or anti-PRP2 antibodies (affinity purified from serum 139, 300µl; lane 7). Lane 8: RNA extracted from anti-PRP2 immunoprecipitate, from 9 µl of reaction in lane 4. Lane 9: RNA from supernate from immunoprecipitation in lane 7 (equivalent of 1 µl of splicing reaction). Lane 10: RNA extracted from anti-m_3G immunoprecipitate from supernate (9µl splicing-reaction-equivalent) shown in lane 9. Lane 11: same RNA as in lane 3, but purified and then cleaved with oligonucleotide C plus *E. coli* RNase H (section 2.4.24). M: markers, with sizes indicated in nucleotides. IVS.E2-lariat intermediate, IVS-lariat intron product, P-pre-mRNA, m-mRNA, E1-exon 1. The arrow on the right hand side of the figure indicates the 3' portion of RNA after cleavage with oligonucleotide C.


Total
Immunoprecipitations
pre-mRNA
RP Δ3 RP Δ3 RP RP RP Δ3
oligo
− − + + + + + +
antibodies
− α m3G αPRP2 αPRP2
IVS.E2
IVS
P
E2
E1
m
E1
622
527
404
309
242
238
217
201
190
180
160
147
122
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90
76
1 2 3 4 5 6 7 8 9 10 11 M

dependent upon spliceosome formation, since it did not occur when the Δ3B pre-mRNA was used as substrate (lane 8). Anti-PRP2 antibodies did not immunoprecipitate exon 1. The control experiment presented in lane 9 shows that the supernatant from the immunoprecipitation in lane 7 contained the same amount of 3' portions of pre-mRNA as the starting material (lane 3), and these were still co-precipitable by anti-m_3G antibodies (lane 10). The fact that anti-PRP2 antibodies co-precipitated only the 5' half of the pre-mRNA provides evidence that PRP2 protein is associated, in spliceosomal complexes with this portion of the RP51A transcript.

Given that in this experiment the 3' half of the pre-mRNA was more or less quantitatively degraded by an endogenous nuclease activity failure to detect immunoprecipitation of this molecule by antibodies against PRP2 protein must make this conclusion tentative.

CHAPTER 6

DISCUSSION

This thesis describes experiments aimed at understanding the involvement of the PRP2 protein in pre-mRNA splicing in *Saccharomyces cerevisiae*

The most important reason for choosing this organism for the study of pre-mRNA splicing was the availability of well characterised splicing mutants strains, including *prp2*. This allowed the cloning of the PRP2 gene, in this laboratory (Lee *et al*, 1984) and by others (Last *et al*, 1984). This, in turn, facilitated an immunological approach to understanding the involvement of the PRP2 protein in RNA splicing, since it allowed the construction of β-galactosidase fusion proteins, for raising antisera to PRP2 protein, in rabbits. The availability of *prp2* strains of *S. cerevisiae* also allowed the development of an assay system for the activity of PRP2 protein in RNA splicing.

The first part of this work involved the generation and characterisation of antibodies to PRP2 protein. The general approach taken was to construct fusions between *E. coli* proteins and different regions of PRP2 protein, and to use these fusion proteins to raise antisera against PRP2 protein. In all, five different fusion proteins were constructed of which three (FP2.2, FP2.3 and FP2.5) were used to raise antisera against PRP2 protein. Together, these fusion proteins contain regions covering the whole of PRP2 protein, with only small regions of overlap (figure 3.1). Injection of each of the fusion proteins into rabbits resulted in the production of antisera which specifically recognised PRP2 protein, both on Western blots and under immunoprecipitation conditions. However, the different antisera had widely different strengths of reaction with PRP2 protein; antisera raised against FP2.2 produced weak responses compared to the antisera against FP2.3 and FP2.5. It is possible that the C-terminal

region of PRP2 protein, present in FP2.2 is a poor immunogen, in rabbits. Antibodies raised against FP2.3 and FP2.5 were used to attempt to detect a metazoan homologue of PRP2 protein, by probing western blots of HeLa cell extracts; no HeLa proteins were specifically recognised by the anti-PRP2 antisera in such experiments (data not presented).

The second major part of the work was over-expression of PRP2 protein in various systems, development of an assay system for PRP2 protein activity in pre-mRNA splicing, (based on the work of Lustig *et al* 1986), and partial purification of the protein. Initial studies of overexpression of PRP2 protein used a high copy number yeast plasmid (pJDB2076), which expressed PRP2 protein constitutively (section 4.1.1). It was found, however, that this plasmid caused growth problems in cells which carried it, and this appeared to be due to the high constitutive levels of PRP2 protein in these cells. Because expression of PRP2 protein, using this plasmid, was not reliable, two further plasmids, in which expression of PRP2 protein was under the control of the yeast *GAL*1 upstream activator sequence, were constructed. These constructions, one based on a low copy number vector and the other on a high copy number vector, allowed expression of PRP2 protein to be regulated by the carbon source upon which the cells were grown. The low copy number construct, pBM-PRP2, gave an approximately 10-fold increased level of expression of PRP2 protein under inducing conditions. The high copy number construct, pKV-PRP2, which also contained a strong promoter (the yeast *PGK* promoter) gave a 100 to 200-fold increased level of expression under inducing conditions, although expression of PRP2 protein was not fully repressed under conditions (growth on glucose as carbon source) designed to repress transcription from the *PGK* promoter. Nevertheless, cells carrying pKV-PRP2 facilitated identification of the PRP2 protein, and were a useful enriched source of PRP2 protein, for partial purification.

PRP2 protein was expressed in *E. coli* using an expression vector, pDR540, which contains the tac promoter. However, in contrast to the high expression of β-galactosidase-PRP2 fusion proteins (section 3.1), the expression of full-size PRP2 protein was considerably lower than in yeast cells containing pBM-PRP2, under inducing conditions, and was furthermore

not consistent, from experiment to experiment. The cause of this problem was probably rapid degradation of PRP2 protein. Because of this problem, E. coli cells carrying pDR-PRP2 were not, used as a source of PRP2 protein for other studies.

PRP2 protein was also expressed by in vitro transcription followed by in vitro translation (section 4.3). For the purpose of these studies the PRP2 gene was subcloned into pSPT18, a vector containing a bacteriophage T7 promoter. PRP2 mRNA was produced by in vitro transcription using T7 polymerase, and this RNA was translated in a rabbit reticulocyte extract. Full sized PRP2 protein was produced in these experiments, but did not show any detectable activity in RNA splicing.

An assay system for PRP2 protein activity in RNA splicing was developed, based upon the work of Lustig et al (1986). This system depends upon the inactivation of splicing extracts from prp2 strains of yeast, by heating. The experiments shown in section 4.4 demonstrate that the splicing activity of the system depended strongly upon the amount of PRP2 protein present, with very low background activity in the absence of added wild type PRP2 protein.

The PRP2 protein was partially purified from extracts of yeast cells carrying pKV-PRP2. The purification procedure, which consisted of sequential steps of centrifugation, ammonium sulphate precipitation and heparin agarose columnfractionation, resulted in considerable purification of the protein, which retained activity in RNA splicing. Other experiments, using anti-PRP2 antibodies for immunoaffinity purification, resulted in purification of small amounts of PRP2 protein. However, the purified PRP2 protein was not active in RNA splicing, due, presumably, to the harsh conditions necessary to efficiently elute the protein from the antibody column (data not presented).

The third major part of the work consists of experiments to investigate the interaction of PRP2 protein with splicing factors and with spliceosomes. It has been shown (Lin et al, 1987; Cheng and Abelson, 1987) that PRP2 protein is not required for the early steps in spliceosome

assembly, and it was suggested that it is a factor 'extrinsic' to the spliceosome. Prior to the experiments presented in Chapter 5 of this thesis, no direct physical association of PRP2 with either spliceosomes or with other spicing factors had been demonstrated although it appears to be functionally associated with another factor, b_n (Lin *et al* 1987). Two other *PRP* gene products, PRP4 and PRP8 proteins, have been shown to be associated with snRNPs (Lossky *et al*, 1987; Banroques and Abelson, 1989; Peterson-Bjorn *et al*, 1989), and it was therefore of interest to determine whether PRP2 protein was also associated with snRNPs.

The approach taken to investigating PRP2 protein interactions with snRNPs and spliceosomes was to use affinity purified anti-PRP2 antibodies to immunoprecipitate PRP2 protein, and to determine whether any snRNPs or spliceosomes were co-precipitated. Using this technique it was not possible to detect any interactions between PRP2 protein and yeast snRNPs. It is possible that such interactions are very weak, or transient and require more sensitive techniques to detect them. This result is consistent with evidence (section 5.2.3) suggesting that PRP2 protein does not associate with splicing complexes until all the U snRNPs are probably already bound. It is also consistent with the results of Cheng and Abelson (1987), who found that in heat inactivated prp2 splicing extracts, spliceosomal complexes formed which contained all the U snRNPs, although heat inactivation of a snRNP-associated prp2 protein might not necessarily prevent its interaction with spliceosomes.

It has, however, been possible, using anti-PRP2 antibodies, to detect co-precipitation of pre-mRNA and splicing intermediates from *in vitro* splicing reactions. This co-precipitation occurs only under conditions in which spliceosomal complexes can be formed, indicating that PRP2 protein interacts with pre-mRNA only during RNA splicing. It was also shown (section 5.2.2) that heat inactivated prp2 protein does not associate with spliceosomes. This result independently confirms that the co-precipitation of pre-mRNA observed with anti-PRP2 antibodies is dependent upon PRP2 protein. These observations and direct co-precipitation of gel-purified spliceosomes are the first demonstration that PRP2 protein interacts directly with spliceosomes rather than acting indirectly in splicing by, for

example, covalently modifying splicing factors, prior to their assembly into spliceosomes.

Anti-PRP2 antibodies co-precipitate pre-mRNA and splicing intermediates, but not products, from an *in vitro* splicing reaction. This result indicates that PRP2 proteinfirst associates with the spliceosome prior to step 1 of the splicing reaction and is present throughout step 1. It is difficult to determine at which point it dissociates from the spliceosome: the lack of co-precipitation of splicing products may indicate that it leaves before step 2, or immediately upon completion of this step. Post-splicing complexes, which contain lariat intron product, complexed with snRNPs have been detected (Konarska and Sharp, 1987); the mRNA remains in an ribonucleoprotein complex which does not contain snRNPs . The PRP2 protein does not appear to associate with either complex, although it is possible that PRP2 protein does associate with them, but that the epitopes which ere bound by anti-PRP2 antibodies are not accessible, in these complexes. This possibility can, in general, not be excluded wherever anti-PRP2 antibodies fail to co-precipitate RNA. It would be desirable to confirm such results using an independent technique for demonstrating the presence of PRP2 protein in spliceosomes.

The experiments demonstrating co-precipitation of RP51A pre-mRNA and splicing intermediates with anti-PRP2 antibodies also revealed that a species (X, in figure 5.2) was preferentially co-precipitated. The 3' end of this species, and of a similar species in actin pre-mRNA, map between the UACUAAC sequence and the 3' splice site. In both RP51A and actin genes, there is a sequence in this region which is very T rich (13/19 nucleotides in actin, 12/15 nucleotides in RP51A). It is likely that during *in vitro* transcription, the bacteriophage polymerase pauses or terminates in this region, due to the limiting amounts of UTP in the transcription reaction (section 2.4.23).

It ws shown that X was capable of undergoing step 1 of the splicing reaction. More detailed analysis of this region in RP51A pre-mRNA revealed that transcripts with less than approximately 32 nucleotides 3' to the UACUAAC sequence are preferentially co-precipitated. This figure is

close to the minimum of 29 nucleotides which was reported to be required for step 1 of the splicing reaction to occur (Rymond et al, 1987).

The preferential co-precipitation of these transcripts with PRP2 protein is explained by the results of the investigation of which specific splicing complexes PRP2 protein is associated with. In the experiment shown in section 5.2.3, four complexes (1, 2, 2' and 3) were observed which, based on (a) the relative electrophoretic mobilities of the complexes, (b) the kinetics of their appearance and (c) the RNA species associated with them , appear to correspond to the complexes A1, A2-1, A2-2 and B observed by Cheng and Abelson (1987). However, in the absence of data on the snRNP composition of the complexes such comparisons must be approached with caution, due to the different gel systems and substrates employed. The species X appeared to accumulate in complex 1, and anti-PRP2 antibodies co-precipitated pre-mRNA predominantly from this complex. Although they observed a slightly different pattern of complex formation, Rymond et al (1987) also found that oligo A-cleaved pre-mRNA was trapped in the most slowly migrating complex. Thus, the relatively inefficient co-precipitation of full length transcripts is explained by the fact that the RNA in complex 1 is a small proportion of the total RNA in the in vitro splicing reaction. This is because pre-mRNA is present in this complex for a very short time, the complex being rapidly converted to the active spliceosome. Species like X, by contrast, which have less than 32 nucleotides 3' to the UACUAAC sequence are trapped in this complex and therefore efficiently co-precipitated by anti-PRP2 antibodies.

The results presented in section 5.2.3 define the initial point of interaction of PRP2 protein with spliceosomal complexes to be the transition from complex 2 to complex 1. While slightly different patterns of complex formation have been seen in other experiments, in no case has PRP2 protein been observed to associate with the fastest migrating (and first appearing) complex on the gel. As noted above, it is possible that PRP2 protein is associated with these complexes, but PRP2 epitopes are not available for antobody binding in these particular complexes. However, since it has been shown (Cheng and Abelson, 1987) that such early

complexes can form in the absence of active PRP2 protein, this possibility is considered unlikely.

By using an oligonucleotide plus endogenous RNase H to cleave the pre-mRNA in the intron it has been possible to separate a fragment of spliceosomes containing the 5' splice sitefrom a fragment containing the UACUAAC box and 3' splice site. Following cleavage of pre-mRNA in in vitro splicing extracts, degradation of a substantial fraction of the population of 3' portions of the molecule occurs, with the result that there is an excess of free 5' portions. A fraction of both portions is m_3G-precipitable. The 3' portions are clearly not all physically associated with 5' portions since anti-PRP2 antibodies do not co-precipitate detectable quantities of the 3' portion, while they strongly co-precipitate the 5' portion. In contrast to this result, when spliceosomes formed on poly A-containing pre-mRNA are purified by immunoprecipitation with antibodies to yeast poly A-binding protein, and are then cleaved by a similar procedure, the two fragments of the pre-mRNA appear to remain associated (not presented). This difference is possibly due to the fact that the immunoprecipitations involve long incubations in buffers containing high salt and EDTA concentrations, in the presence of excess competitor RNA. Rymond and Rosbash (1986) showed that the association between the two fragments of the cleaved spliceosome was disrupted under non-denaturing gel electrophoresis conditions similar to the immunoprecipitation conditions used here.

The results of experiment presented in section 5.2.4 show that the 5' portion of spliceosomal complexes co-precipitate with PRP2 protein indicating that, at least in complex 1, PRP2 protein is associated with this region of the spliceosome. An additional weaker association of PRP2 protein with a subcomplex attached to the 3' portion of the cleaved RNA cannot be excluded, as certain interactions may be disrupted upon cleavage and dissociation of the spliceosome. Although it as not possible to clearly identify the cleaved lariat intermediate species, the lack of co=precipitation of exon 1 might suggest that (subject to the same reservation) that PRP2 protein is associated with the intron. It is

tempting to speculate that the PRP2 protein is associated with complexes at the 5' splice site.

The current state of knowledge regarding the involvement of PRP2 protein in RNA splicing may be summarised as follows. PRP2 protein is a factor required for RNA splicing in S. cerevisiae. It is not associated with any snRNP and is not required for the early stages of spliceosome formation. It binds to spliceosomal complexes, possibly in association with the b_n factor, at a specific stage of spliceosome assembly, the transition from complex 2 to complex 1. In complex 1, at least, it is associated with the region of the pre-mRNA containing the 5' splice site. PRP2 protein is present thoughout step 1 of the splicing reaction, and leaves the spliceosome at some point subsequent to completion of this step, possibly when splicing complexes dissociate upon completion of step 2.

Given this information, it is possible to speculate upon the function of the PRP2 protein in pre-mRNA splicing. The facts that PRP2 protein is not associated with any snRNP, that it is not required for the early stages of splicing complex formation and only enters the spliceosome when all the U snRNPs are probably already present, suggest that PRP2 protein does not have a major structural role in the spliceosome, but may rather be closely connected to the catalytic functions of splicing complexes. Its possible association with the 5' splice site suggests that it is involved in step 1 of the splicing reaction, and the presence of the zinc finger structure in the protein suggests that it may bind directly to the pre-mRNA. One possibility is that PRP2 protein is the endonuclease responsible for 5' splice site cleavage, in step 1 of the splicingreaction. The nuclease activities in group I and group II RNA splicing have been shown to reside in RNA molecules (Cech and Bass, 1986), and it is often assumed that, given the apparent similarities in mechanism between group I, group II and pre-mRNA splicing, that this will also be the case in the latter. Thus it is expected that the catalytic activity will reside in one of the U snRNA species, with the associated proteins playing a role similar to the maturases of groups I. This is, however, only a supposition and it is possible that the catalytic activities in pre-mRNA splicing have been taken over by proteins, such as PRP2 protein.

A second possibility is that PRP2 protein is responsible for 5' splice site selection, or the exact positioning of the catalytic apparatus relative to the 5' splice site. It has been shown that there exist proteins in mammalian systems which enhance the correct binding of U1 snRNP to the 5' splice site (Mayeda et al, 1986; Zapp and Berget, 1989). Other possible functions for PRP2 protein include bringing the 5' splice site and branchpoint sequences together, during step 1 of the reaction.

With regard to future investigations of the function of PRP2 protein, there are a number of important questions which remain to be answered. Firstly, it is of interest to know whether PRP2 protein directly contacts the pre-mRNA. This could be answered by UV-crosslinking studies, although such studies have so far failed to observe any such direct interaction (E. Whittaker, personal communication). If such studies did detect binding of PRP2 protein to RNA, they could be followed up by mapping of the binding site by T1 RNase digestion of pre-mRNA in spliceosomes, followed by immunoprecipitation with anti-PRP2 antibodies. It would also be of interest to determine whether ATP is required for PRP2 binding to spliceosomes. This could be investigated by allowing splicing complexes to form in heat inactivated prp2 extracts, depleting the extracts of ATP using glucose and hexokinase and then adding PRP2 protein in the presence and absence of fresh ATP, followed by immunoprecipitation using anti-PRP2 antibodies. ATP binding to PRP2 protein could be examined using radio-labelled ATP analogues which contain groups which make covalent cross links to proteins, when irradiated with UV light.

Other studies might focus on the interactions of PRP2 protein with other splicing factors. This might be approached by isolating and characterizing trans-acting suppressors of prp2 mutants. Such studies have been initiated (D. Jamieson, personal communication).

An alternative possible approach to the identification of yeast proteins which bind to PRP2 protein, would be to use immobilised PRP2-β-galactosidase fusion proteins, if these can be obtained in a native form, as affinity columns for ^{35}S-labelled yeast proteins. This approach is more direct than the method of co-precipitation used in section 3.3, and would

allow analysis of which region of PRP2 protein interacts with such proteins.

A different method for analysis of the function of PRP2 protein would be site directed mutagenesis of the protein. Such a mutation, of an amino acid which is a crucial part of the putative zinc finger structure, has been generated, and fails to complement the prp2 defect in vivo (E.Whittaker, G.Anderson and J.Beggs, personal communication).

Finally, the technique of cleavage of spliceosomes into two portions, followed by co-precipitation of the 5' portion with anti-PRP2 antibodies, allows the investigation of general aspects of spliceosome architecture. In particular, it would be of interest to determine which snRNPs are co-precipitated with the 5' portion of the RNA in such experiments.

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